Remarks

Finality of the Office Action

All rejections are newly presented in the Office Action mailed January 30, 2007. Nonetheless the Office Action is designated as a final rejection. It is respectfully asserted that the finality of the rejection is premature. Applicants request that the finality be withdrawn to permit more meaningful examination to occur.

Applicants responded to the prior rejections in good faith in a manner that the PTO found persuasive. All prior rejections were withdrawn. Applicants should be given at least one opportunity to freely and meaningfully respond to the new rejections prior to finality being imposed.

Specification

A new copy of the abstract is provided on the preceding page. This abstract was part of the application as originally filed in the PCT and does not constitute new matter. It should already be part of the application.

Rejection of Claims 3-5 and 22-24 under 35 U.S.C. §112. first paragraph

Claims 3-5 and 22-24 stand rejected as not described in the specification; the claims are alleged to contain impermissible new matter. This rejection is respectfully traversed.

The rejection states that paragraph 52 of the specification "does not specifically disclose the ranges set forth in claims 3-5 and 22-24 and applying an electric field intradermally." Final Office Action at page 5, lines 17-18. Claim 1 was amended to recite "intradermally" applying an electric field. This recitation is not objected to as new matter in claim 1. Claims 3-5 are original and have not been amended. Thus each of the individual recitations of claims 3-5 ("1 to 100 pulses," "1 microsecond to 5 seconds," and "10-5,000 V/cm") are supported at least by the original claims which are part of the specification. Similarly in claim 22, the recitation of "applying between 1 and 20 pulses of between 500 and 2,000 V/cm and between 10 and 1000 microseconds" are original to the claim. Thus the issue appears to be the combining of the "intradermal" limitation with the other specific recitations.

Applicant notes that the teaching of intradermal application of the electric field is taught generally in the specification *inter alia* at paragraph 36 where pin electrodes are taught. Fields of uniform or pulsed quality of various field strengths are taught generally at paragraph 35. It is respectfully submitted that any of the possible parameters generally taught in the specification as filed can be combined without violating the new matter prohibition. Nonetheless, to expedite examination and focus the issues on those which are most important, applicants have amended the claims to recite the parameters which the examiner points to

as specifically disclosed in connection with intradermal administration of electric field. These are supported at paragraph 52.

Withdrawal of this rejection is respectfully requested.

Rejection of Claim 42 under 35 U.S.C. §112, second paragraph

Claim 42 is rejected as indefinite. The term "wounded tissue is at the wound's border" is allegedly relative, which therefore makes it indefinite. Moreover, the rejection alleges that "it is not apparent how a wounded tissue can only be at the wound's border." Claim 42 has been amended to clarify that the electric field is applied at the wound's border so that the claim does not appear to state that the wounded tissue is only at the wound's border. It is respectfully submitted that this amendment clarifies the meaning of the claim. Withdrawal of this rejection is respectfully requested.

Rejection of Claims 1, 2, 3, 5, 6, 7, 19, 21, 41, and 44 under 35 U.S.C. § 103(a)

Claims 1, 2, 3, 5, 6, 7, 19, and 21 stand rejected as unpatentable over, Zhang et al., U.S. 6,972,013 taken with Glasspool-Malone. This rejection is respectfully traversed.

To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. M.P.E.P. §2143. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in applicant's disclosure. In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). The present rejection fails in the second criteria.

The PTO cites Zhang as teaching non-invasive *in vivo* electroporation to skin. Zhang does not specifically teach intradermal application of the electric field. In fact, Zhang teaches away from any invasive (intradermal) electroporation at column 14, lines 19-65, mentioning pain, fear, and risk of infection associated with invasive electroporation. Glasspool-Malone is cited for teaching the application of electric field intradermally. Neither reference teaches, however, the intradermal application of an electric field to wound tissue and the effect on healing. Both references employ normal, healthy skin in their experiments. Because of the great differences between wound tissue and normal healthy skin, one of ordinary skill in the art would not have had a reasonable expectation of success in practicing the claimed invention on wound tissue.

Using common knowledge and common sense, we know that wound tissue is very different in its

properties from normal skin. Lokmic (Exhibit 1; Wound Rep. Reg. 2006, 14: 277-288) meticulously documents and characterizes many differences between normal skin and wound tissue. Lokmic used histochemical localization of hypoxia to characterize one such difference. Using Hypoxyprobe-1™ which is a substituted 2-nitroimidzaole which binds only to cells that have oxygen concentration less than 14 μM, Lokmic detected hypoxic cells and regions of hypoxia. As shown in Figure 2, normal skin did not label with the probe, whereas wounded tissue displayed changing regions of labeling as the wound healed from 3 days to 3 weeks. Labeled regions in the wound tissue included granulation tissue, and blood vessels, fibrin clot, new epidermis, hair follicles, myofibroblasts, and macrophages.

Lokmic also characterized the differences in the vascular volume of normal skin versus wound granulation tissue. Figure 3 shows that at the peak (7 days) the wound tissue had about 16 % vascularity while the normal skin had only about 3 %.

Lokmic also characterized the differences in the proliferation of normal cells and wounded tissue, using a Ki67 antibody. The difference between day 0 (normal cells) and day 3 (wound tissue) is pronounced, and is quantitated in Figure 4C. At day 3 there are about 125 cells per square meter and at day 0 less than 5 cells per square meter.

Lokmic further characterized the differences between normal skin cells and wounded tissue by measuring apoptosis using a TUNEL label (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling). As shown in Figure 5, the number of apoptotic cells in wound granulation tissue at 2-6 weeks was markedly greater than in normal skin cells as shown at day 0 (Fig. 5C) and the negative control (Fig. 5A). At 3 weeks about 12 apoptotic cells per square meter were observed. At day 0 less than 1 cell per square meter was observed.

Lokmic also characterized the difference in vascular endothelial growth factor-A (VEGF-A) expression using immunohistochemical localization. Again, as seen in Figure 6, the difference between normal skin and the wounded skin is clear. Similarly, vascular endothelial growth factor receptor-2 (VEGFR-2) labeling indicated differences in expression between the two types of tissue as shown in Figure 7. Labeling for VEGF-A was found in wound tissue, vascular sprouts, myofibroblasts, smooth muscle cells, endothelium of arterioles, epidermal cells, hair follicles, and connective tissue. VEGFR2 was observed in wound tissue in vascular sprouts, macrophages, and smooth muscle cells. VEGFR2 is a receptor involved in angiogenesis. See page 284.

Differences in labeling of the two tissue types were also seen when sections were labeled for alpha-smooth muscle actin (α-SMA) (Figure 8) and transforming growth factor-beta (TGF-β) (Figure 9). TGF-β regulates cell proliferation, differentiation, adhesion, migration, Extracellular Matrix (ECM) production, and vascular remodeling. See page 284. Thus Lokmic shows that normal skin and wounded skin are different by a number of important biological and biochemical criteria including connective

tissue cell proliferation, apoptosis, hypoxia, marker expression, vascular volume, and remodeling. These confirm our common knowledge and common sense experiences of the differences between normal skin and wound tissue.

Because of the very large and important differences between wound tissue and normal skin, one of ordinary skill in the art would not have had a reasonable expectation that a process that may have been successful in normal skin would be successful in wound tissue. Moreover, the extent of effect on wound healing could not have been predicted.

Indeed, the combination of electric pulses and delivery of growth factors provides greater than additive results. As shown in Exhibit 2 (Lee et al., "Electroporatic delivery of TGF-β1 gene works synergistically with electric therapy to enhance diabetic wound healing in db/db mice," *J. Invest. Dermatol.* 123: 791-198, 2004) the effect of this combination on wound healing is far greater than additive. The effect of the combination on the percentage of actively proliferated epidermal cells at region 2 of the wound edge of the epithelium at day 3 post-wounding is dramatic. Electric pulses alone yielded about a 20 % yield, intradermal growth factor-encoding plasmid alone yielded about a 32 % yield, and the combination yielded about a 70 % yield. See Figure 4B. Similarly the effect of the combination therapy on angiogenesis in the granulation tissue at the center of the wound bed at day 7 post-wounding is far greater than additive. The endothelial cell count with electric pulses alone is undetectable, and with intradermal administration of growth factor-encoding plasmid alone it is about 2-3 cells per field. In contrast, the combination treatment yielded about 24 endothelial cells per field. See Figure 7. Again, by this criterion, the combination yields an effect that is far greater than additive. Lee states that "indeed the electric effect and gene effect work synergistic[ally] in the genetically diabetic model." Abstract, last line.

Lee et al.'s conclusion is reinforced by Sattinger and Goldsmith in their column "Clinical Snippets, " *J. Invest. Dermatol.* 123: vi, 2004 (Exhibit 3): "The combination of electric pulses and delivery of TGF-β1 plasmid provided an innovative synergism to treat diabetic wound healing in the db/db (diabetic) mouse. The findings by Lee and co-workers may have a significant implication for clinical applications."[1]

"A greater than expected result is an evidentiary factor pertinent to the legal conclusion of obviousness ... of the claims at issue." *In re Corkill*, 711 F.2d 1496, 226 USPQ 1005 (Fed. Cir. 1985). Evidence of a greater than expected result may also be shown by demonstrating an effect which is greater than the sum of each of the effects taken separately (i.e., demonstrating "synergism"). *Merck & Co. Inc. v. Biocraft Laboratories Inc.*, 874 F.2d 804, 10 USPQ2d 1843 (Fed. Cir.), *cert. denied*, 493 U.S. 975

[1] The publication date of Lee et al. is after any of the priority dates to which the applicant is entitled according to pages 2 and 3 of the Final Office Action.

(1989).

Thus the combination of references would not have rendered the invention obvious to one of skill in the art at the time of the invention because there are so many differences between wounded tissue and normal skin that one of ordinary skill in the art would not have had a reasonable expectation of success that the intradermal electric field administration to normal skin of Glasspool-Malone would be successful when applied to wounded tissue. Moreover, even if, *arguendo,* the *prima facie* case remains, the very positive and synergistic effect of the treatment on wound healing could not have been anticipated and rebuts the *prima facie* case of obviousness.

Withdrawal of this rejection is therefore requested in view of this evidence.

Rejection of Claims 1,4, 15, and17- 24 under 35 U.S.C. § 103(a)

Claims 1, 4, 15, and 17- 21 stand rejected as unpatentable over Zhang taken with Glasspool-Malone as applied above, and further in view of Bureau taken with Ruben. This rejection is respectfully traversed.

Bureau is cited as teaching electroporation to deliver a transgene to cells of the tissue being treated for regeneration of vascularization by angiogenic factors produced by transgenes encoding, *e.g.,* growth factors, using certain parameters of pulses and timing. The PTO states, "[h]owever, Bureau does not specifically teach using the method to treat a wound in a patient."

Ruben is cited as teaching that growth factors can be used to treat wounds burns, and skin disorders. In addition, Ruben is cited as teaching delivery of polynucleotides encoding a growth factor to wounds. Moreover, the PTO points to page 69 of Ruben as teaching electroporation in gene therapy. However, the teaching of Ruben pertains to *in vitro* treatment of cells by electroporation. In its encyclopedic lists of possible modes of treatment, Ruben teaches that a "nucleic acid is introduced into a cell prior to administration in vivo of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation....." Paragraph 610, emphasis added. The electroporation-treated cells are subsequently delivered to the patients as protein delivery agents. "The resulting recombinant cells can be delivered to a patient by various methods known in the art." Paragraph 611. Ruben does not teach the application of electroporation to a wound to stimulate healing, as required by the claimed methods. Ruben does not teach or suggest that the electroporation should be applied to the patient's wound rather than to cells in culture.

Nothing in this expanded combination of references, as in the binary combination of Glasspool-Malone and Zhang discussed above, teaches the intradermal administration of electroporation to wound tissue. One of ordinary skill in the art would not have had a reasonable expectation of success in such an endeavor because of the large number of fundamental differences between wound tissue and normal skin.

Moreover, the results obtained upon actually combining intradermal delivery of electroporation and polynucleotide encoding a growth factor to wound tissue are far greater than additive, demonstrating strong synergy. As above, evidence of these two facts negate or rebut the *prima facie* case of obviousness.

Withdrawal is respectfully requested.

Rejection of Claims 1 and 14under 35 U.S.C. § 103(a)

Claims 1 and 14 stand rejected as unpatentable over Zhang and Glasspool-Malone further in view of Arbeit (US 6838430). This rejection is respectfully traversed.

Glasspool-Malone and Zhang are already discussed above. The two primary references do not form a *prima facie* case of obviousness because neither teaches the use of intradermal administration of electroporation to wound tissue. One of ordinary skill in the art would not have had a reasonable expectation that this combination of teachings would be successful based on intradermal administration of normal skin because normal skin and wound tissue are so different. The teachings of Lokmic in Exhibit 1 demonstrate that biologically the two types of tissue are extremely different, confirming what common sense tells us. Thus there would have been no reasonable expectation of success in wound tissue based on normal skin. Moreover, the combination of the two treatments (intradermal electroporation and polynucleotide encoding growth factor) yields synergistic effects. See Lee et al., Exhibit 2.

Arbeit does not remedy these defects in the primary references. Arbeit is cited as teaching the use of a nucleic acid encoding the elected species of growth factor, HIF-1 alpha, to stimulate wound healing in a patient with a wound. This teaching does not remedy the defects in the *prima facie* case. The primary references do not provide a reasonable expectation of success that intradermal administration of an electric field to wound tissue would increase wound healing. Thus the three-reference rejection also fails to provide a *prima facie* case of obviousness. Withdrawal of this rejection is respectfully requested.

Rejection of Claims 1 and 42-43under 35 U.S.C. § 103(a)

Claims 1 and 42-43 stand newly rejected as unpatentable over Zhang and Glasspool-Malone further in view of newly cited Braddock (US 6838430). This rejection is respectfully traversed.

Braddock is cited as teaching descriptive biology regarding the wound healing process. Specifically, the PTO quotes a portion that states that proliferation of cells and synthesis of extracellular matrix components is regulated by certain growth factors secreted by the wound border cells. The rejection asserts, totally without basis, that one of skill in the art "would have been motivated to combine the teaching [to] improve the transfection of the transgene and increase the activity of mediators secreted from the wound border cells." The teaching of Braddock does not change the basic defects with the two primary references. One of ordinary skill in the art would not have had an expectation of success that

intradermal application of an electric field to wound tissue in combination with administration of a polynucleotide encoding a growth factor would successfully increase wound healing. Wound tissue is so different from normal skin, that one of ordinary skill would not have been able to reasonably extrapolate from the behavior of one to the other. See Lokmic, Exhibit 1. Even if there had been a reasonable expectation of success, one of skill in the art would not have expected the degree of success. The two treatments when combined yield a greater than additive effect which is described in the literature as "innovative synergism." See Sattinger and Goldsmith, Exhibit 3.

Withdrawal of this rejection is respectfully requested.

Respectfully submitted,

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EXHIBIT 1

Time course analysis of hypoxia, granulation tissue and blood vessel growth, and remodeling in healing rat cutaneous incisional primary intention wounds

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Manuscript received: August 4, 2005
Accepted in final form: January 12, 2006

DOI:10.1111/j.1743-6109.2006.00122.x

ABSTRACT

Hypoxia and the development and remodeling of blood vessels and connective tissue in granulation tissue that forms in a wound gap following full-thickness skin incision in the rat were examined as a function of time. A 1.5 cm-long incisional wound was created in rat groin skin and the opposed edges sutured together. Wounds were harvested between 3 days and 16 weeks and hypoxia, percent vascular volume, cell proliferation and apoptosis, α-smooth muscle actin, vascular endothelial growth factor-A, vascular endothelial growth factor receptor-2, and transforming growth factor-β_1 expression in granulation tissue were then assessed. Hypoxia was evident between 3 and 7 days while maximal cell proliferation at 3 days (123.6 ± 22.2 cells/mm^2, $p < 0.001$ when compared with normal skin) preceded the peak percent vascular volume that occurred at 7 days ($15.83 \pm 1.10\%$, $p < 0.001$ when compared with normal skin). The peak in cell apoptosis occurred at 3 weeks (12.1 ± 1.3 cells/mm^2, $p < 0.001$ when compared with normal skin). Intense α-smooth muscle actin labeling in myofibroblasts was evident at 7 and 10 days. Vascular endothelial growth factor receptor-2 and vascular endothelial growth factor-A were detectable until 2 and 3 weeks, respectively, while transforming growth factor-β_1 protein was detectable in endothelial cells and myofibroblasts until 3–4 weeks and in the extracellular matrix for 16 weeks. Incisional wound granulation tissue largely developed within 3–7 days in the presence of hypoxia. Remodeling, marked by a decline in the percent vascular volume and increased cellular apoptosis, occurred largely in the absence of detectable hypoxia. The expression of vascular endothelial growth factor-A, vascular endothelial growth factor receptor-2, and transforming growth factor-β_1 is evident prior, during, and after the peak of vascular volume reflecting multiple roles for these factors during wound healing.

Healing of skin wounds involves complex cellular and molecular responses which, under normal conditions, eventually result in closure of the wound gap and the formation of scar tissue.[1] Knowledge of the natural history of wound healing is essential before any pathological processes can be identified and studied, and before patients can be advised about the expectation of how a wound might heal or the wisdom, or otherwise, of revisional surgery. Scarring is inevitable following wounding but the final appearance at maturation is known to vary considerably according to such parameters as age, underlying systemic disease (e.g., diabetes), site on the body, heredity, race, and circumstances of healing such as primary or secondary repair, infection or inflammation, and wound tension.[2] Obtaining the best possible scar following surgical intervention is through elimination of dead space between the wound edges and avoidance of hematoma, foreign body suture material, infection, and tension in the wound. Large wound gaps can lead to delayed, prolonged, or sometimes exaggerated healing with excessive cellular proliferation, scaring, and contracture. Prolonged vascularity, in particular, is the hallmark of a scar that is likely to remain prominent and cosmetically unfavorable.

Granulation tissue formation, characterized by blood vessel and fibroblast proliferation and migration from adjacent intact tissues and extracellular matrix (ECM)

DAB	3',3-Diaminobenzidine
EC	Endothelial cell
ECM	Extracellular matrix
H&E	Hematoxylin & eosin
HRP	Horseradish peroxidase
PBS	Phosphate buffered saline solution
α-SMA	α-Smooth muscle actin
SMC	Smooth muscle cell
TGF-β_1	Transforming growth factor-β_1
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VEGF-A	Vascular endothelial growth factor-A
VEGFR-2	VEGF receptor-2

synthesis in a wound gap, occurs in response to various polypeptide growth factors. Injured tissue is also subject to hypoxia as the circulation becomes compromised. Hypoxia is a potent stimulator of vascular endothelial growth factor (VEGF) synthesis.[3] Subsequent angiogenesis leads to the migration of new capillaries into the provisional matrix between the wound edges—initially a fibrin clot that is replaced by newly synthesized connective tissue. It is also possible that endothelial blood borne progenitors, angioblasts, may contribute to new vessel formation.[4] The new connective tissue is synthesized by myofibroblasts, a fibroblast cell capable of migrating and characterized by contractile features particular to those of smooth muscle cells (SMCs) making this cell type an intermediate between a fibroblast and a SMC.[5]

Under the influence of factors including VEGF,[6] placental-derived growth factor,[7] and fibroblast growth factor 2[8] the endothelial cells (ECs) proliferate, form angiogenic sprouts and migrate through the ECM. The EC sprouts then form a vessel lumen through various mechanisms[9] regulated in part by VEGF[10] and integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$.[11] The vessels undergo stabilization by recruiting supporting pericytes and SMCs under the influence of platelet-derived growth factor-B and its receptor,[12,13] with maturation under the regulation of angiopoietins and their receptors.[14]

The peculiar characteristic of wound healing is that the newly formed capillaries and surrounding connective tissue are remodeled through apoptosis before a return of tissue homeostasis by the formation of a largely avascular scar.[15,16] Cellular signals that determine the fate of such vessels are currently unknown. It has been suggested that hypoxia has a role in activation of apoptosis,[17] however the evidence that hypoxia could play a main role in activating and maintaining the apoptotic process leading to vessel and tissue remodeling in the wound[18] requires further investigation.

To date, no studies have been reported that examine specifically the relationship between hypoxia and quantitative vascular and connective tissue development and remodeling across the wound gap during primary repair of a rat skin incisional wound over a long time period. The aim of this study was to establish a relationship between the presence of hypoxia (visualized by hypoxyprobe-1) and connective tissue and vascular development and remodeling of incisional wound granulation tissue in adult rats by examining the percent vascular volume density, cell proliferation, and apoptosis over a time frame of 3 days to 16 weeks. This model of wound healing was chosen as it represents a state following major groin surgery, and reflects a situation closely resembling that of a human. Furthermore, the expression of VEGF-A, its receptor VEGFR-2, α smooth muscle cell actin (α-SMA), and transforming growth factor β_1 (TGF-β_1) has also been investigated as these have been considered to be among the most important regulators of angiogenic development and remodeling.

MATERIALS AND METHODS

Six male Sprague–Dawley rats per group (ARC, Perth, WA, Australia), weighing between 280 and 320 g, were used for each time point group (3, 7, and 10 days and 2, 3, 4, 6, 8, and 16 weeks). One wound from each of the 4, 8, and 16 weeks became infected and was excluded from the study. In addition, another five rats were used to harvest unwounded skin (time point zero). All experimental and medical procedures were approved by the Animal Ethics Committee of St. Vincent's Hospital, Melbourne, Australia (AEC number 34/02) and were conducted in accordance with the Australian National Health and Medical Research Council guidelines for the care and maintenance of laboratory animals.

Surgical procedures

The rats were anesthetized with intraperitoneal phenobarbitone (30 mg/kg). Under sterile conditions, a longitudinal 1.5 cm incision was made with a scalpel on the medial aspect of the groin through the skin and the underlying fat pad. The opposed edges of the wounds were sutured together with 10.0 nylon sutures and animals allowed to recover.

The animals were reanesthetized on days 3, 7, and 10; or 2, 3, 4, 6, 8, or 16 weeks after the formation of the wound, with intraperitoneal phenobarbitone (30 mg/kg). A circumferential incision, approximately 1 cm away from the primary incision site, was made and the wound tissue harvested. The specimen was further sectioned into 2 mm thick slices perpendicular to the primary incision. In addition, normal skin (dimensions 1 cm × 1 cm) was excised from the groin of five unwounded rats and used as control skin samples. Following tissue collection, the animals were euthanized by intraperitoneal injection of Lethobarb® (200 mg/kg, Virbac, Peakhurst, Australia).

Histological procedures

Wound tissue was immersion fixed in 10% buffered formol saline for 12 hours, followed by a wash in 0.1 M phosphate buffered saline solution (PBS) for a further 12 hours, and dehydrated through a graded series of alcohols and histolene for paraffin embedding. Three micrometer thick sections were cut from all blocks at each time point and mounted on poly-L-lysine-coated slides. Sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome and some sections from each block were reserved for immunohistochemistry.

Hypoxia analyses

One and a half hours before wound tissue collection, two animals from each of the 3, 7, and 10 days, and the 2, 3, and 4 weeks groups were injected via the tail vein, with 1.5 mL of hypoxyprobe-1 (Hypoxyprobe™, Natural Pharmacia Intl. Inc., Belmont, MA) at a dose of 100 mg/kg, then euthanized and tissue processed for routine histology. Hypoxyprobe-1 is a substituted 2-nitroimidazole whose chemical ingredient is pimonidazole hydrochloride.[19,20] It is distributed to all tissues via the circulation[20] and it binds only to cells that have oxygen concentrations less than 14 μM (this is equivalent to a pO_2 of 10 mmHg at 37 °C) making it a suitable reagent to detect hypoxic cells and identify the regions of hypoxia.[21]

Before euthanasia, at tissue harvest, in addition to the wound tissue, a sample of liver tissue and normal skin was

also collected from hypoxyprobe-1-injected rats as a positive control[22] and from hypoxyprobe-1-free animals as a negative control.

To immunohistochemically localize the bound hypoxyprobe-1, tissue sections were immersed in 10 mM sodium citrate buffer, pH 6.0, at 90 °C for 30 minutes. The sections were then cooled to room temperature, washed in PBS and incubated in 3% H_2O_2 in methanol for 10 minutes. The nonspecific binding was blocked by incubating sections in 10% rabbit serum for 20 minutes. Excess serum was removed and hypoxyprobe-1 antibody (Natural Pharmacia Intl. Inc., 1:200 dilution) applied overnight at 4 °C, followed by incubation with biotinylated rabbit anti-mouse secondary antibody (Dakocytomation, Carpinteria, CA) diluted 1:200 in PBS for 30 minutes. The sections were then washed in PBS and signal detected by streptavidin-horseradish peroxidase (HRP; Dakocytomation, 1:400 dilution) and visualized by 3'3-diaminobenzidine (DAB; Dakocytomation). The liver sections were additionally blocked for endogenous biotin by 0.1% avidin–biotin blocking solution.

Morphometric analysis

To visualize the blood vessels in the wound granulation tissue, dewaxed tissue sections were pretreated with protease K solution (Dakocytomation) for 8 minutes. The sections were washed in distilled water, quenched in 3% H_2O_2 for 15 minutes, and washed in 0.1 M tris-buffered saline, pH 7.5 and incubated in biotinylated *Bandeiraea Simplicifolia* lectin (Vector Laboratories, Burlingame, CA, 1:100 dilution) for 30 minutes. The unbound lectin was washed away with 0.1 M tris-buffered saline, and bound lectin detected by streptavidin–HRP–DAB as described above. The negative control included omission of the *Bandeiraea Simplicifolia* lectin from the reaction. To determine the percent vascular volume, stained wound sections were viewed under ×20 magnification with a test grid eyepiece. A random starting field was selected for counting, after which every 20th field was counted through out the wound granulation tissue and fibrin clot. The number of points that overlay blood vessels was divided by the total number of points counted (the sum of granulation tissue connective tissue, blood vessel, and fibrin points per section) and expressed as a percentage.[23] Morphometric counts were made independently by two observers (Z.L. and G.M.) blinded to treatment and the counts compared to ascertain that no more than 10% difference existed between counts from the two observers on the same tissue sections.

Immunohistochemical procedure for cell proliferation

To detect proliferating cells, the sections were stained with Ki67 antibody (Dakocytomation) as previously described.[24] To allow quantitation of the proliferating cells in the incisional cutaneous wound granulation tissue and fibrin clot only, the dividing cells in hair follicles, epidermis, sweat glands, uninjured tissue, panniculus carnosus, and the subcutaneous adipose tissue were excluded from the count. The sections were viewed under ×40 magnification and images captured via a digital video camera (TK C1480E, JVC, Yokohama, Japan) attached to a microscope. The entire tissue area was measured and a series of fields within that area randomly and systematically sampled so that 5% of total area of the tissue was counted. The proliferating cells in the tissue were counted using an electronic stepping stage (H128, Prior Scientific, Rockland, MA) and the Computer Assisted Stereological Toolbox (CAST System, Olympus Denmark A/S, Albertslund, Denmark). The proliferating cell number was expressed as the mean number of proliferating cells/$mm^2 \pm$ standard error of the mean (SEM) for each wound.

Detection of apoptotic cells

The apoptotic cells in wound sections were detected as previously described.[25] Rat lymph node was used as a positive control. To count the apoptotic cells between the wound edges within granulation tissue only, the apoptotic cells in hair follicles, fibrin clot, epidermis, sweat glands, uninjured tissue, macrophages containing terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive remnant DNA in their cytoplasm, panniculus carnosus, and subcutaneous adipose tissue were excluded from the count. The macrophages containing TUNEL-positive DNA remnants also contained hemosiderin, an iron pigment of golden brown refractive appearance collected by macrophages when they phagocytose and breakdown effete red blood cells in the zone of hemorrhage.[26,27] Apoptotic cells were counted as described for the proliferating cells and expressed as the mean number of apoptotic cells per $mm^2 \pm$ SEM.

Immunohistochemical analysis for growth factors, receptors, and α-SMA

To immunohistochemically examine the expression of VEGF-A, VEGFR-2, α-SMA, and TGF-β_1 in the incisional wound tissue, the sections were pretreated in 10 mM citric acid buffer, pH 6.0 at 95 °C for 30 minutes, then washed in PBS. To block endogenous peroxidase all sections were immersed in 10% H_2O_2 in methanol for 20 minutes then washed in PBS for 5 minutes.

For VEGF-A labeling, the sections were blocked in 10% goat serum for 30 minutes, followed by an overnight incubation at 4 °C in polyclonal goat anti-mouse antibody specifically directed against VEGF-A (kind gift from Genentech Inc., San Francisco, CA) diluted 1:500 in PBS. Sections were washed in three changes of PBS and incubated in HRP-conjugated rabbit anti-goat secondary antibody (1:400, Dakocytomation) for 30 minutes. The bound complex was detected by DAB.

To detect VEGFR-2 expression, the sections were blocked in 10% rabbit serum and incubated in 1:50 dilution of goat anti-mouse VEGFR-2 antibody (R&D Systems, Minneapolis, MN) overnight at 4 °C. The bound antibody was detected by biotinylated rabbit anti-goat secondary antibody (1:200, Dakocytomation) for 1 hour. The VEGFR-2 labeling was visualized by streptavidin–HRP and DAB.

To detect myofibroblasts, α-SMA was detected by incubating dewaxed sections first in 10% rabbit serum for 30 minutes, followed by mouse anti-human α-SMA antibody (1:200, Dakocytomation) at 4 °C overnight. The bound antibody was detected by biotinylated rabbit

anti-mouse antibody (1 : 400, Dakocytomation) and streptavidin–HRP–DAB.

TGF-β_1 was detected by incubating sections first in 10% rabbit serum then in mouse anti-human TGF-β_1 antibody (1 : 100, R&D Systems) overnight at 4 °C. Sections were then washed in PBS and incubated in biotinylated rabbit anti-mouse secondary antibody (1 : 400, Dakocytomation) for 30 minutes. The complex was visualized by streptavidin–HRP and DAB. For each antigen, the negative control consisted of a section incubated in the same species-specific nonimmune immunoglobulin instead of the primary antibody.

Statistical analysis

For vascular morphometric, cellular proliferation and apoptosis studies, the results were reported as mean ± SEM for the total number of observations. Statistical comparison between the groups was performed using one-way ANOVA and the Bonferroni multiple comparisons tests. The results were considered statistically significant when $p < 0.05$.

RESULTS

Three-day wounds were characterized by deposition of a hemorrhagic fibrin clot (containing platelets, and leukocytes including macrophages), in the wound gap (Figure 1A). The superficial fibrin clot was dried out (forming an escar) while the underlying fibrin clot became a provisional matrix to invading connective tissue cells, angiogenic hyperpermeable blood vessels and macrophages (collectively referred to as the granulation tissue), and the severed edges of the epidermis. By 7 days, the epidermal layer had completely migrated underneath the escar (Figure 1B) while no clear margins could be distinguished between the newly formed granulation tissue and the preexisting vascularized connective tissue.

By 10 and 14 days the epidermal tissue started to remodel as the epithelial layer covering the wound gap thinned out to resemble that of normal skin. Between 14 and 21 days (Figure 1C) granulation tissue could be identified; however, hyperpermeable blood vessels appeared fewer in number. From 21 days to 16 weeks (Figure 1C and D) the newly deposited collagen (scar) stained pale blue with Masson's trichrome thus being easily distinguished from collagen in uninjured tissue.

Immunohistochemical localization of hypoxia in incisional wounds

Negative control tissue (Figure 2A) and normal skin (Figure 2B) did not label for hypoxyprobe-1. In contrast many hepatocytes (Figure 2C) were positive for hypoxyprobe-1. At 3 days postwounding, the granulation tissue surrounding the fibrin clot is a narrow rim at the wound edges characterized by high cellularity, sprouting blood vessels, arterioles, venules, and capillaries surrounding the fibrin clot which all labeled positive for hypoxyprobe-1 (Figure 2D). The immediately adjacent epidermal layer, hair follicles, and fibrin clot were also positive for hypoxyprobe-1 (Figure 2E).

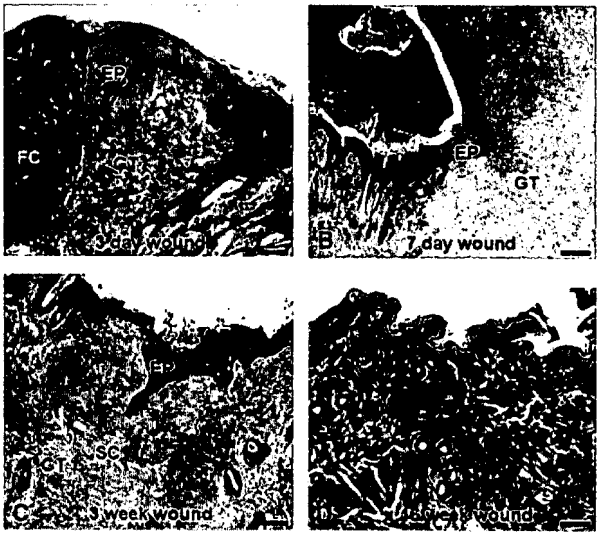

Figure 1. Histology of a healing incisional wound. (A) Three-day wound tissue characterized by the deposition of a fibrin clot (FC), into the wound gap, migrating epidermis (EP) at the wound edge, and formation of granulation tissue (GT) immediately adjacent to the fibrin clot. (B) By 7 days the EP had migrated underneath the escar (E) separating it from the GT. (C) At 3 weeks the EP was remodeling and pale blue scar (SC) tissue was evident replacing the GT. (D) By 16 weeks only a pale blue SC (between lines) was evident at the wound site. Stain: Masson's trichrome; scale bars = 150 μm.

Similar observations were made at 7 days with hypoxia evident in blood vessels and cells found only in the granulation tissue (Figure 2F) within and immediately adjacent to the fibrin clot. In the healthy epidermis (associated with uninjured tissue) and the new epidermis that had migrated across the wound gap only those cells closest to the skin surface labeled with hypoxyprobe-1. At 10 days hypoxyprobe-1 staining was confined to the cells (myofibroblasts and macrophages) and blood vessels of the granulation tissue (Figure 2G), hair follicles and epidermis immediately adjacent and migrating directly over the wound. At 2 and 3 weeks labeling was confined to scattered granulation tissue cells, the new epidermis and hair follicles (Figure 2H). At 4 weeks (Figure 2I) no hypoxyprobe-1 labeling was evident in wound granulation tissue.

Vascular volume of granulation tissue

A significant difference was observed between the percent vascular volume of the normal skin and the 3- ($p < 0.05$), 7- ($p < 0.001$), and 10-day ($p < 0.01$) wound granulation tissue (Figure 3A). The highest percent vascular volume density in the wound granulation tissue was observed at 7 (15.83 ± 1.10%) and 10 days (14.48 ± 2.58%) (Figure 3A and B). The 7-day percent vascular volume was also significantly different from the 16-week value ($p < 0.05$). After 10 days the percent vascular volume declined (Figure 3A and C). Sixteen weeks after wounding the percent vascular volume (6.36 ± 1.23%) was slightly higher than in normal skin; however, this was not statistically significant.

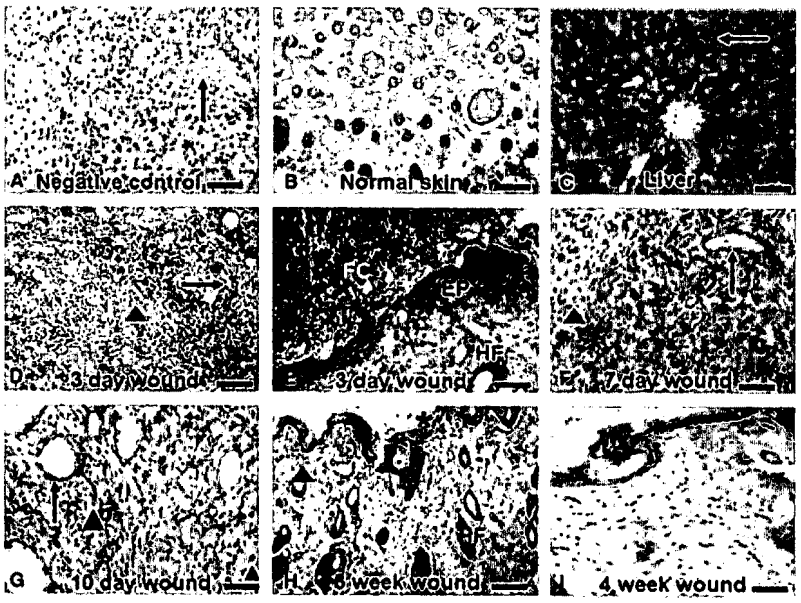

Figure 2. Immunohistochemical localization of hypoxia (hypoxyprobe-1 monoclonal antibody) in incisional wounds and liver. All tissues shown are from animals injected with hypoxyprobe-1. (A) Negative control 7-day wound granulation tissue without labeling (compare with F). Arrow indicates blood vessel. (B) No labeling is present in normal skin from an adjacent groin region to the incisional wound. (C) Liver tissue showing numerous positively labeled cells (arrow). (D) In 3-day wounds, hypoxia was identified in the granulation tissue cells (arrowhead) and blood vessels (arrow). (E) Three-day wound showing hypoxyprobe-1 labeling in fibrin clot (FC), new epidermis (EP), and hair follicles (HF). (F) At 7 days, hypoxia was still detected in the cells (arrowhead) and blood vessels (arrow) of the granulation tissue. (G) At 10 days hypoxia persisted in the granulation tissue cells, myofibroblasts (arrowhead on left), macrophages (arrowhead on right), and blood vessels (arrow). (H) At 3 weeks hypoxia was confined to individual cells associated with the scar granulation tissue (arrowhead) and the upper layers of EP (asterisk) and HF. (I) By 4 weeks no hypoxia was observed in any region of the wound; scale bar = 50 μm for (A,C,F,G) and (I) 100 μm for B,D,E, and H.

Cellular proliferation in granulation tissue

Negative control tissue (Figure 4A) did not show any evidence of positive Ki67-labeling, and only rare Ki67-positive cells were seen in normal rat skin. A significant level of proliferation was observed in 3-day wound granulation tissue, particularly in the capillary endothelium (Figure 4B) and myofibroblast population. Proliferating cells were also noted in the new epidermis migrating over the wound gap although epidermal tissue was not included in the counts. From day 10, as healing progressed, proliferation was confined to the myofibroblast population in the granulation tissue and epidermal cells. Blood vessels of the granulation tissue showed no evidence of proliferation after 7 days.

Maximal cell proliferation in granulation tissue (principally myofibroblasts and capillary endothelium; Figure 4C) was observed in the 3-day wound tissues (123.6 ± 22.2 cells/mm^2), after which time the number of proliferating cells declined rapidly until 3 weeks (13.8 ± 4.9 cells/mm^2). The decline thereafter was gradual and at 16 weeks (3.9 ± 0.7 cells/mm^2) was only slightly higher than the number of proliferating cells observed in normal skin (2.2 ± 0.7 cells/mm^2). The number of dividing cells seen at 3 days was significantly higher when compared with the 3- ($p < 0.05$), 4- ($p < 0.01$), 6-, 8-, and 16-week ($p < 0.001$) granulation tissue and normal skin ($p < 0.001$).

Apoptosis in incisional wounds

Apoptotic cells in the wound granulation tissue were initially confined to the fibrin clot and closely associated hair follicles. The majority of apoptotic cells from 7 days to 4 weeks were myofibroblasts and fibroblasts of the granulation tissue. Occasionally an apoptotic blood vessel was seen in the 2- and 3-week granulation tissue (Figure 5B compare with negative control Figure 5A). Vessel apoptosis was characterized by the loss of EC and pericytes and collapse of the vessel, leaving only basement membrane remnants. The presence of "ghost" basement membranes in wound granulation tissue of 2- and 3-week wounds was the only evidence of previously existing vessels. As the epidermis repaired and involuted toward the normal skin surface, an occasional TUNEL-positive cell was observed in the epidermis; however, this was a rare event.

The highest number of apoptotic cells (Figure 5C) was observed in the 3- and 4-week wound granulation tissue (12.1 ± 1.3 and 9.6 ± 0.9 cells/mm^2, respectively). The number of apoptotic cells/mm^2 at both time points was significantly higher from the values recorded for normal skin ($p < 0.001$, for both 3 and 4 weeks), 3- ($p < 0.001$, and $p < 0.01$, 3 and 4 weeks, respectively), 7- ($p < 0.001$ and $p < 0.01$, 3 and 4 weeks, respectively), 10-day ($p < 0.05$, for both 3 and 4 weeks), 6- ($p < 0.05$, for both 3 and 4 weeks), 8- ($p < 0.001$, $p < 0.01$, for 3 and 4 weeks, respectively), and 16-week wounds ($p < 0.01$, for both 3 and 4 weeks). After 3 weeks, the number of apoptotic cells declined gradually and at 16 weeks (1.1 ± 0.2 cell/mm^2) was only slightly higher than the number of apoptotic cells in normal skin (0.48 ± 0.28 cell/mm^2). This difference was not statistically significant.

VEGF-A expression

VEGF-A protein was not evident in negative control tissue (Figure 6A) or normal skin (Figure 6B). However, immunohistochemical detection of VEGF-A protein was

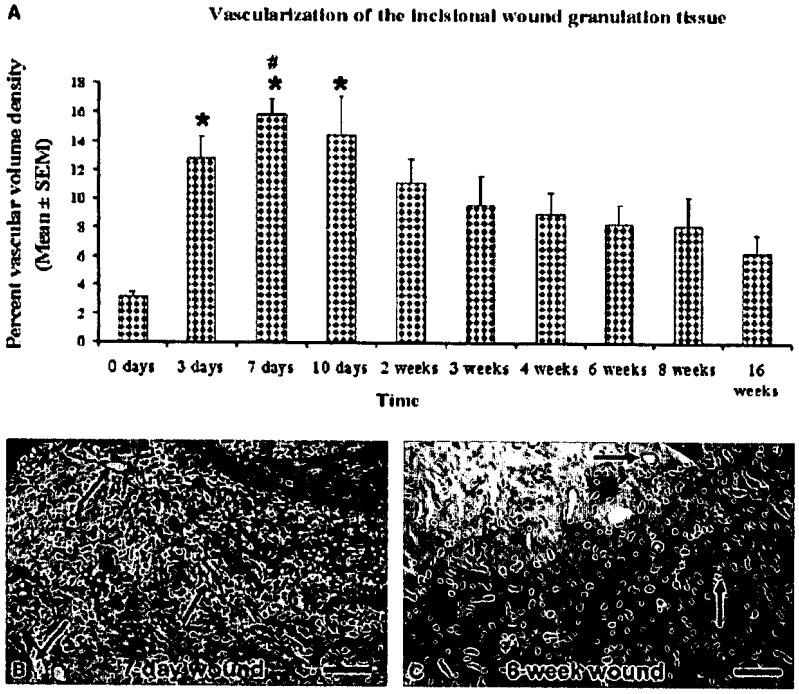

Figure 3. Percent vascular volume of wound granulation tissue. (A) Percent vascular volume of the incisional wound granulation tissue over 16 weeks presented as mean (± SEM). Note that day 0 represents percent vascular volume of normal skin. *Significantly greater percent vascular volume in 3- ($p < 0.05$), 7- ($p < 0.001$), and 10-day ($p < 0.01$) wound granulation tissue compared with normal skin. #The 7-day wound percent vascular volume was significantly greater than the 16-week value ($p < 0.05$). (B), (C) *Bandeiraea Simplicifolia* lectin staining of incisional wound granulation tissue blood vessels (arrows) at (B) 7 days and (C) 42 days. Note smaller and less numerous blood vessels at the later time point; scale bar = 100 μm.

observed throughout wound granulation tissue. This was particularly evident at 3 (Figure 6C), 7, and 10 days (Figure 6D) and 2 weeks (Figure 6E) when VEGF-A was prominent in vascular sprouts, myofibroblasts, SMCs in the tunica media and endothelium of arterioles within and at the periphery of the granulation tissue. VEGF-A was also expressed by migrating epidermal cells and hair follicles adjacent to the injured area. Significant binding of the VEGF-A antibody was also observed in the connective tissue (when compared with the negative control). This expression gradually declined between 3 and 6 weeks, although an occasional connective tissue cell still showed some labeling at, and beyond, 6 weeks (Figure 6F).

VEGFR-2 expression

Negative control tissue (Figure 7A) and normal skin did not show VEGFR-2 labeling (Figure 7B). At 3 (Figure 7C) and 7 days, the VEGFR-2 antibody was identified in vascular sprouts and macrophages scattered throughout the wound granulation tissue, and in ECs and SMCs of arterioles and venules associated with the granulation tissue. By 10 days two specimens still showed VEGFR-2 expression in blood vessels, while by 2 weeks, only one specimen contained VEGFR-2 positive cells. At 3 weeks, only an occasional VEGFR-2 positive inflammatory cell was identified in the granulation tissue. VEGFR-2 immunostaining was not observed in the 4-, 6- (Figure 7D), 8-, and 16-week wound tissues.

α-SMA expression

Negative control tissue did not label positive for α-SMA (Figure 8A). However, α-SMA-labeled SMCs in the tunica media of blood vessels and the majority of pericytes surrounding capillaries in uninjured normal skin (Figure 8B). Blood vessel pericytes and smooth muscle cells in granulation tissue labeled similarly wherever granulation tissue was evident (Figure 8C, D, and F). In addition, a small number of myofibroblasts were labeled in 3-day granulation tissue. The number of labeled myofibroblasts increased markedly in 7- and 10-day wounds (Figure 8D and E). Labeling of myofibroblasts at 14 days and at later time points declined and although blood vessel labeling persisted, the total number of vessels in the granulation tissue declined after 14 days and therefore the α-SMA labeling in blood vessels was also reduced (Figure 8F).

TGF-β_1 expression in granulation tissue

Negative control tissue did not show TGF-β_1 labeling (Figure 9A). TGF-β_1 labeling was also not evident in normal skin except for the superficial layers of the epidermis (Figure 9B). In the 3- (Figure 9C and E), 7- (Figure 9F), and 10-day wound tissue TGF-β_1 was prominent in endothelium of the sprouting blood vessels, macrophages, myofibroblasts of granulation tissue, in the migrating new epidermal cells and in external root sheaths of both the injured and healthy hair follicles. At 2 and 3 weeks (Figure 9D and G), TGF-β_1 expression had subsided and was only detected in myofibroblasts (Figure 9 G) of the granulation tissue and the external root sheaths of hair follicles and the epidermis. From 4 to 16 weeks no TGF-β_1 signal was observed in the granulation tissue, with an exception of occasional macrophages and white blood cells. The absence of TGF-β_1 expression in myofibroblasts (collagen-secreting, migrating fibroblasts) after 3 weeks in the wound granulation tissue corresponds to the loss of α-SMA labeling in myofibroblasts. Most myofibroblasts probably

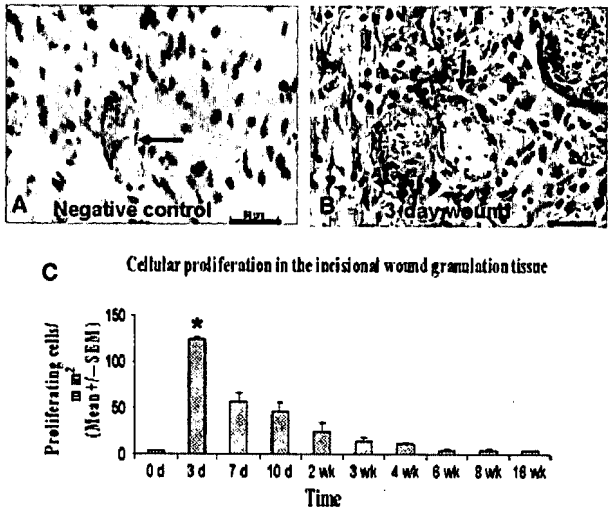

Figure 4. Ki67-labeled wound tissue. (A) Negative control tissue of 3-day wound granulation tissue. No positive labeling is evident in the blood vessels (arrow) or interstitial cells of the granulation tissue. (B) Ki 67-labeled wound granulation tissue at 3 days showing proliferating cells within the capillary endothelium (arrow); scale bar=50 μm. (C) Number of proliferating cells/mm^2 in incisional wound granulation tissue over a 16-week period presented as mean (± SEM). Note that day 0 represents the number of proliferating cell /mm^2 in normal skin. *Number of dividing cells seen at 3 days was significantly higher when compared with the 3- ($p < 0.05$), 4- ($p < 0.01$), 6-, 8-, and 16-week ($p < 0.001$ for all three time points) granulation tissue and normal skin ($p < 0.001$).

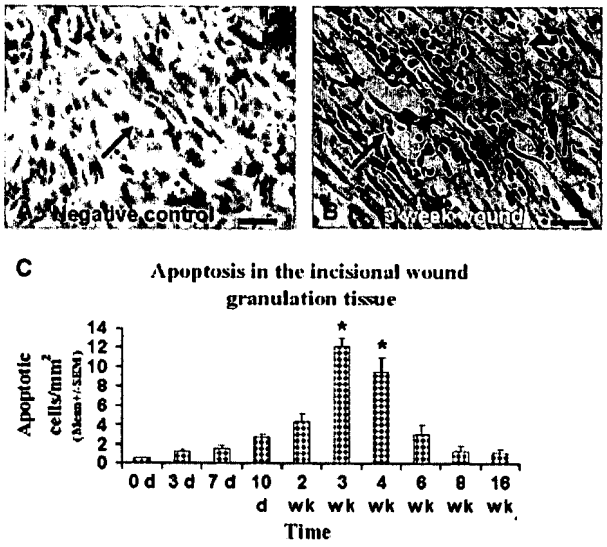

Figure 5. TUNEL-labeled apoptotic cells in the wound tissue. (A) Negative control tissue of 3-week wound granulation tissue showing no labeling in blood vessel endothelium (arrow) or interstitial connective tissue cells. (B) TUNEL-labeled endothelial cell (arrow) and connective tissue cell (arrowhead) in 3-week wound granulation tissue; scale bar = 50 μm. (C) Number of apoptotic cell profiles/mm^2 in incisional wound granulation tissue over a 16-week period presented as mean (± SEM). *Number of apoptotic cells/mm^2 in 3- and 4-week granulation tissue was significantly higher than the values recorded for normal skin ($p < 0.001$, for both 3 and 4 weeks), 3-day ($p < 0.001$ and < 0.01, 3 and 4 weeks, respectively), 7-day ($p < 0.001$ and < 0.01,3 and 4 weeks, respectively), 10-day ($p < 0.05$, for both 3 and 4 weeks), 6- ($p < 0.05$, for both 3 and 4 weeks), 8- ($p < 0.001$ and $p < 0.01$, for 3 and 4 weeks, respectively), and 16-week wounds ($p < 0.01$, for both 3 and 4 weeks).

undergo apoptosis and the predominant connective tissue cells remaining after 4 weeks are mature fibroblasts which do not label with α-SMA or TGF-β_1. The binding of TGF-β_1 antibody to the ECM (Figure 9C, D, F, and G) was noted (when compared with negative control) throughout the 16-week study, although the intensity of this staining decreased between 4 and 16 weeks and probably reflects the presence of TGF-β_1 bound to the wound ECM. Normal skin ECM (Figure 9B) did not show TGF-β_1 labeling.

DISCUSSION

By examining rat incisional wound granulation tissue from 3 days to 16 weeks after wounding, we have obtained an overall representation of relationships between hypoxia, percent vascular volume density, cellular proliferation and apoptosis, VEGF-A, VEGFR-2, α-SMA, and TGF-β_1 in the tissue.

Wound healing involves rapid and significant cellular proliferation in the granulation tissue as the highest number of proliferating cells was observed at 3 days, followed by an increase in the percent vascular volume density of this tissue (maximal at 7 days). The dividing cells comprised myofibroblasts and endothelial cells of angiogenic capillaries. The later were found not only at the tip of the migrating capillaries but also distally to the tip suggesting that the partial formation of new vessels is by longitudinal extension.[9] The rapid cellular proliferation and vessel development in the granulation tissue coincided with the presence of hypoxia in the wound.

In addition to hypoxia, a concurrent presence of VEGF-A, VEGFR-2, and TGF-β_1 protein illustrated by strong immunohistochemical labeling in wound granulation tissue supports previous observations that hypoxia and related transcription factors such as hypoxia-inducible factor-1 may be involved in the expression of trophic factors including VEGF-A,[28,29] VEGFR-2 on angiogenic vessels[30] and up-regulation of TGF-β_1.[31,32] Previous wound studies suggest that hypoxia as shown by pimonidazole hydrochloride adduct formation was not evident at day 1[18] despite the presence of significant growth factor expression at this early time point. However, the study of Albina et al.[33] indicates that although pimonidazole hydrochloride adduct formation may not be apparent in wounds at 24 hours, hypoxia inducible factor-1α expression was maximally expressed at 6 hours postwounding and was still present at 1 and 5 days postwounding.

Evidence of hypoxia in wound tissue declined after 2 weeks. Subsequently the granulation tissue reduces to a narrow scar tissue becoming largely avascular and

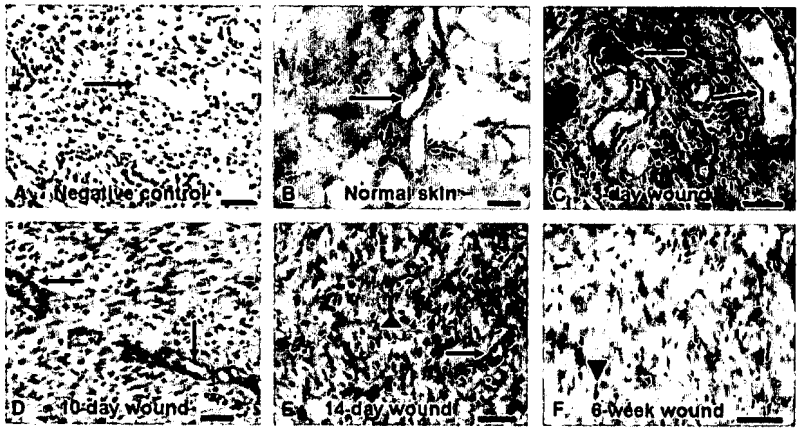

Figure 6. Immunohistochemical localization of VEGF-A in incisional wound granulation tissue. (A) Negative control slide of 3-day wound granulation tissue without any evidence of positive labeling in blood vessels (arrow). Compare with (C). (B) Normal groin skin showing no positive labeling in blood vessels (arrow). (C) Three-day wound granulation tissue showing positive VEGF-A labeling in blood vessel endothelium (arrows). (D) VEGF-A labeling in 10-day wound granulation tissue within the endothelium of capillaries (arrows). (E) Fourteen-day wound granulation tissue showing persistent VEGF-A labeling in myofibroblasts (arrowhead) and capillary endothelium (arrow). (F) At 6 weeks VEGF-A labeling is considerably reduced with in the wound only being evident in the occasional connective tissue cell (arrowhead); scale bar all panels = 50 μm.

acellular. It is unclear if the absence of hypoxyprobe-1 labeling in granulation tissue/scar at late time points (after 2 weeks) is due to lack of perfusion of hypoxyprobe-1 through reducing blood vessels numbers in this area or alternatively that the developing scar tissue is thin enough to be perfused adequately by adjacent vessels. However, the absence of hypoxyprobe-1 labeling in neighboring uninjured tissue indicates adequate perfusion of oxygen in blood vessels in these regions.

TGF-β regulates many tissue transformation processes including cell proliferation, differentiation, adhesion, migration, ECM production, and embryonic development.[34]

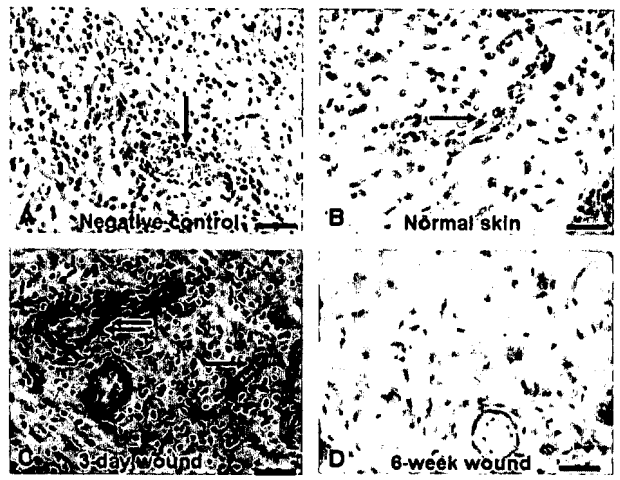

Figure 7. Immunohistochemical localization of VEGFR-2 in incisional wound granulation tissue. (A) Negative control slide of 3-day wound without evidence of VEGFR-2 labeling in blood vessels (arrow). Compare with (C). (B) Normal groin skin showing no VEGFR-2 labeling in dermal blood vessels (arrow). (C) Three-day wound showing positive VEGFR-2 labeling in capillary endothelium (arrow) and arteriole smooth muscle actins (double arrow). (D) At later time points (6 weeks) VEGF-R2 labeling did not occur in the wound scar; scale bar all panels = 50 μm.

Furthermore, of the three TGF-β family members, TGF-β_1 has been implicated in fibrosis and connective tissue and vascular remodeling.[35,36] In the early stages of wound healing it appears from this study that TGF-β_1 is not a deterrent to in vivo angiogenesis as previously suggested[37] as the development of granulation tissue capillaries in the early time points (3, 7, and 10 days) occurs during simultaneous expression of VEGF-A, VEGFR-2, and TGF-β_1. These results also suggest that TGF-β_1 has multiple roles in the development of blood vessels that could possibly encompass formation of solid cords[38] or regulation of basement membrane synthesis during vessel migration into the granulation tissue as was observed with rat liver sinusoidal ECs grown in vitro.[39]

Simultaneous with the expression of VEGF-A at 3, 7, and 10 days, the ECs of new vessels expressed VEGFR-2, a receptor involved in angiogenesis.[40] VEGFR-2 was further observed to be expressed in the SMCs of arterioles associated with the granulation tissue during the early stages of wound healing. As this antibody did not stain the ECs of the capillaries in the normal skin or the SMCs of the arterioles in the uninjured skin, it is unlikely that the antibody is nonspecifically binding to the SMCs of the arterioles in the granulation tissue. Although it has previously been suggested that up-regulation of VEGFR-1 and VEGFR-2 could be involved in in vivo arteriogenesis,[41] that study did not examine the anatomical location of VEGFR-1 and VEGFR-2 during arteriogenesis. No reports have been made on the expression of VEGFR-2 by the arteriolar vascular SMCs during inflammation or the up-regulation of VEGFR-2 in the SMCs of arteries during arteriogenesis. Recently, however, a mouse embryonic precursor cell was identified that can give rise to both ECs and SMCs in vivo and in vitro.[42] The destiny of the precursor cell was dependant on the growth factor it was exposed to, i.e. exposure to PDGF-B lead to the development of SMCs, while exposure to VEGF resulted in endothelial differentiation.[42] Endothelial precursor cells are also known to be able to differentiate into skeletal[43] and cardiac muscle[44] cells. Therefore, it is possible that vascular SMCs of arterioles could have a capacity to transiently express an endothelial marker such as VEGFR-2 if the two cells have a

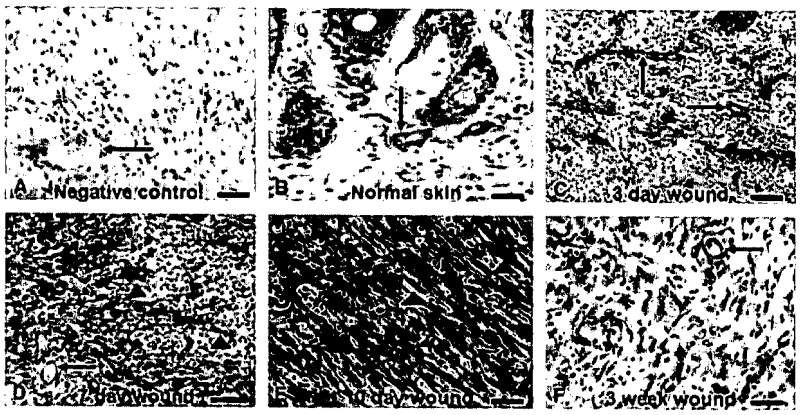

Figure 8. Immunohistochemical localization of α-SMA in wound granulation tissue. (A) Negative control from 10-day granulation tissue with no evidence of α-SMA in blood vessel walls (arrow) or connective tissue cells. (B) Normal groin skin. Note α-SMA labeling in arteriole walls (arrow). (C) Three-day wound showing α-SMA labeling in mural cells of capillaries (arrows) and arterioles of the granulation tissue. (D) Seven-day wound showing myofibroblast α-SMA (arrowheads) labeling in the granulation tissue and within blood vessel walls (arrow). (E) Higher power view of wound granulation tissue at 10 days illustrating extensive α-SMA labeling in myofibroblasts (arrowheads). (F) Twenty-one day wound granulation tissue shows a decrease in α-SMA labeling compared with earlier time points. Labeling has disappeared from the connective tissue cell component suggesting that myofibroblasts no longer form part of theconnective tissue. Some labeling is present in capillary and arteriole walls (arrow) although no more than in normal skin [compare with (B)]; scale bar = 50 μm for (A,B,E and F); 100 μm for (C and D).

common origin. Alternatively, the transient expression of VEGFR-2 in SMCs of arterioles in wound granulation tissue suggests that vascular SMCs express VEGFR-2 to serve an as yet undefined role in arteriolar response to injury and/or inflammation. Further validation of these findings is required.

Following the peak of percent vascular volume density at 7 days, VEGFR-2 expression declines while the number of apoptotic cells in the granulation tissue increases to a maximium at 3 weeks indicating that tissue remodeling is occurring. Both of these events occur as hypoxia in the tissue decreases (as detected by hypoxyprobe-1). Previous studies which examined hypoxic events in skin wounds indicate no hypoxia at day 1[18,33] and maximal hypoxia at days 4–5 that declined thereafter.[18] This time frame of evident hypoxia is a little shorter than that shown in the current study. Haroon et al.[18] have stated that the peak in hypoxia at 4 days corresponds to the initiation of apoptosis and remodeling in the granulation tissue; however, they did not label for apoptotic events in their tissue. In this study, apoptotic cells were labeled and quantitated, showing some apoptotic events occurred during the hypoxic period; however, maximal apoptosis in healing rat incisional wound occurred at 3 weeks when no hypoxia was evident. As apoptosis is a relatively short event, taking less than 24 hours,[45] there is little evidence in this study of a strong association between hypoxia and apoptosis in skin wound granulation tissue further implying that a hypoxic stimulus is not necessary for the majority of apoptotic events. The increase in apoptotic events at 3 weeks may be related to a number of factors including the relative reduction in trophic factors that occurs as the wound reepithelializes and white blood cells disappear from the granulation tissue and/or ECM remodeling.[46,47]

An important question that arises from these studies is whether vessel regression occurs before cellular apoptosis in the connective tissue or vice versa. The results suggest that the decrease in vascular volume (i.e., vessel remodeling through apoptosis) occurs before cellular apoptosis in the connective tissue indicating that loss of blood supply also has a role in wound granulation tissue remodeling. After 4 weeks, the number of apoptotic cells also declined suggesting that tissue remodeling was slowing down. The apoptotic cells in granulation tissue included myofibroblasts, pericytes, and ECs as previously shown.[14,15] The loss of capillaries was further evidenced by the presence of "ghost" basement membranes[48] where vessels once existed.

The peak of apoptosis occurred in the absence of detectable VEGFR-2 expression, confirming previous observations of vessel apoptosis in the absence of VEGFR-2 expression despite the presence of VEGF-A.[49,50] In this study VEGF-A continued to be expressed by myofibroblasts in the remodeling granulation tissue in the absence of VEGFR-2. VEGF-A functions as a survival factor for ECs[50,51] and should, theoretically, prevent in vivo apoptosis of capillary endothelial cells in granulation tissue. However, apoptosis in capillaries was observed when VEGF-A expression was still detectable in myofibroblasts (e.g., 14 and 21 days). This suggests that either the amount of VEGF-A secreted by the myofibroblasts is insufficient to prevent EC apoptosis of some neighboring capillaries, or that the presence of VEGF-A at these time points has a different role, namely in vessel maturation or maintenance of vessel structure.[40] The peak of apoptosis at 3 weeks occurred in the presence of VEGF-A, TGF-β_1, and α-SMA expression by myofibroblasts and in the absence of hypoxia labeling.

The number of apoptotic cells/mm^2 was never as high as the maximal number of proliferating cells in the wound gap although apoptotic events occurred over a longer time frame. It is possible that the clearance of apoptotic cells occurred faster than we could measure in the time points selected, hence the lower counts. In addition, a proportion of apoptotic bodies were present in macrophages (additionally identified by the presence of refractive hemosiderin[27]) that were not included in the counts as more than one apoptotic profile is likely to be included in a single macrophage. There are also a number of potential inaccuracies in interpretation of TUNEL labeling and counting. This includes an inability to detect single-

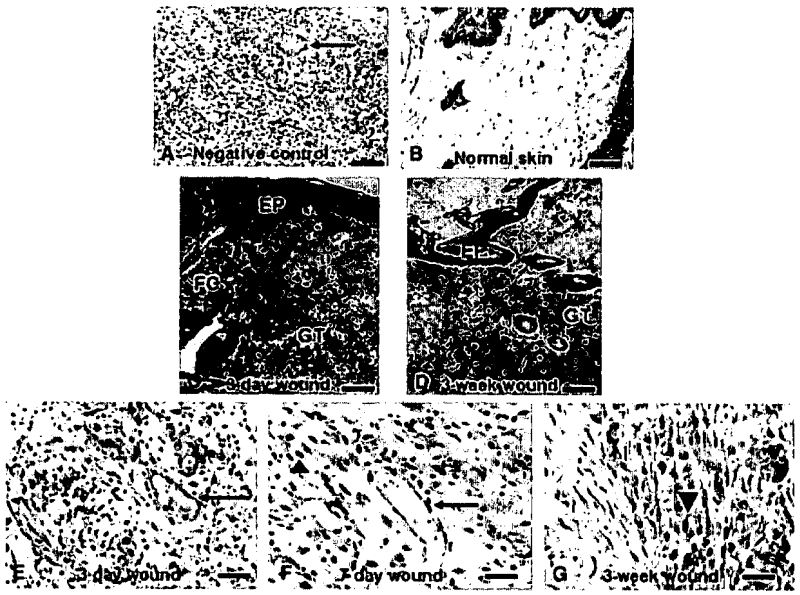

Figure 9. Immunohistochemical localization of TGF-β_1 in wound granulation tissue. (A) Negative control tissue from a 3-day wound showing no TGF-β_1 labeling within the connective tissue or blood vessels (arrow: compare with (E). (B) Normal groin skin. Labeling is not evident within the connective tissue or blood vessels, although some labeling is seen in the superficial layers of the epidermis. (C) At 3 days, TGF-β_1 was located in the migrating epidermis (EP) and granulation tissue (GT) adjacent to the fibrin clot (FC). (D) At 3 weeks TGF-β_1 staining was still evident in the ECM of the GT and EP structures. (E) Higher power of 3-day wound granulation tissue. TGF-β_1 labeling is evident in the capillary endothelium (arrow). (F) At 7 days blood vessels (arrow) and most cells of the granulation tissue (arrowhead) label. (G) Wound myofibroblasts (arrowhead) at 3 weeks showing persistent TGF-β_1 labeling; scale bar = 50 μm for (E–G); 100 μm for (A–D).

stranded DNA breaks[25,52] and the possibility that some necrotic cells also labeled.[53]

Even at 16 weeks, the percent vascular volume is slightly higher than the percent vascular volume density observed in normal skin suggesting that tissue remodeling to form a relatively avascular scar is a gradual process that may take more than 16 weeks, hence any conclusions about wound granulation tissue remodeling should include long-term observations.

We conclude that in incisional wounds, the development of granulation tissue shows a rapid and substantial proliferative response which peaks 3 days after wounding. The peak vascular volume density was observed at 7 days post-wounding immediately following the peak in cell proliferation. The proliferation of connective tissue cells, mainly myofibroblasts, and the blood vessel endothelium occurs in the presence of hypoxia, VEGF-A, and TGF-β_1. The remodeling of granulation tissue occurs soon after, resulting from apoptosis of capillaries and α-SMA-positive myofibroblasts. Maximal apoptosis was at 3 weeks in the absence of detectable hypoxia. Wound remodeling occurs partially in the presence of continued expression of both VEGF-A and TGF-β_1. However, the higher than normal skin percent vascular volume density in 16-week wounds suggest that this remodeling continues at more refined levels and takes more that 16 weeks after wounding to produce a largely avascular scar.

ACKNOWLEDGMENTS

We thank Professor Wayne A. Morrison for critical reading of this manuscript and Mr. Jason Palmer for assistance in the final preparation of the manuscript. This study was supported by the St. Vincent's Hospital Melbourne Research Grant 2003/48 and Microsurgery Foundation Grant awarded to Z. Lokmic. Z. Lokmic is also a recipient of a Melbourne Research Scholarship. We also thank the Experimental and Medical Surgical Unit at St. Vincent's hospital for their help with the animal surgery and care of animals.

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EXHIBIT 2

See related Commentaries on pages vi and xi

Electroporatic Delivery of TGF-β1 Gene Works Synergistically with Electric Therapy to Enhance Diabetic Wound Healing in db/db Mice

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Electrical stimulation (ES) is a therapeutic treatment for wound healing. Electroporation, a type of ES, is a well-established method for gene delivery. We hypothesize that proper conditions can be found with which both electrical and gene therapies can be additively applied to treat diabetic wound healing. For the studies of transforming growth factor-β1 (TGF-β1) local expression and therapeutic effects, full thickness excisional wound model of db/db mice was used, we measured TGF-β1 cytokine level at 24 h postwounding and examined wounds histologically. Furthermore, wound closure was evaluated by wound-area measurements at each day for 14 d. We found that syringe electrodes are more effective than the conventional caliper electrodes. Furthermore, diabetic skin was more sensitive to the electroporative damage than the normal skin. The optimal condition for diabetic skin was six pulses of 100 V per cm for 20 ms. Under such condition, the healing rate of electrically treated wound was significantly accelerated. Furthermore, when TGF-β1 gene was delivered by electric pulses, the healing rate was further enhanced. Five to seven days postapplication of intradermal injection of plasmid TGF-β1 followed by electroporation, the wound bed showed an increased reepithelialization rate, collagen synthesis, and angiogenesis. The data indicates that indeed the electric effect and gene effect work synergistic in the genetically diabetic model.

Key words: diabetic wound healing/electroporation/gene transfer/intradermal injection/TGF-β1 plasmid
J Invest Dermatol 123:791–798, 2004

Wound healing is compromised in diabetic mellitus. The patients have difficulty regeneration the skin barrier, resulting in ulceration and even infection. The impairment has previously been identified as impaired cellular infiltration, collagen, and granulation tissue formation (Brown *et al*, 1997). Several studies have reported that growth factor or its receptor deficiencies relate to diabetes-induced impairment of wound healing. Beer *et al* (1997) demonstrated a reduced expression of PDGF A and B and of A-type receptor in wounded or unwounded diabetic skin. An IGF-1 reduction of 42% in wound fluid and of 48% in serum was observed in diabetes-related impairment (Bitar and Labbad, 1996). A 55% reduction of transforming growth factor-β1 (TGF-β1) expression in diabetic wound fluid has also been shown in the same study.

The administration of exogenous growth factors have been successfully used to accelerate the pathologic wound healing in human and animal models. Topical administration of PDGF-BB-stimulated wound healing in genetically diabetic mice (Greenhalgh *et al*, 1990) and was eventually approved by the FDA for treating diabetic ulcer. A single dose of TGF-β in a collagen vehicle can restore the diabetes-related decrease in tensile strength of collagen (Bitar and Labbad, 1996). Broadley *et al* (1989–1990) also reported that injection of TGF-β-induced accumulation of granulation tissue, and collagen production and maturation. One disadvantage of growth factors, however, are their high expense. Instead of using growth factors, the recent approach is to administer a gene that encodes a growth factor (Yao and Eriksson, 2000). The contribution of growth factor gene to problematic wound healing in animal models appears as successful as the growth factor itself, although no direct comparison has been reported. Subcutaneous injection of interleukin-6 plasmid to mice restores abnormal wound healing (Gallucci *et al*, 2001). In a dermal ulcer model, topical application of PDGF embedded in collagen sponges promotes reepithelialization, wound closure, and new granulation tissue formation (Tyrone *et al*, 2000). In our previous study, intradermal injection of TGF-β1 gene could accelerate wound closure (Chesnoy *et al*, 2003). TGF-β1 is a multifunctional growth factor. It is a chemokine for fibroblasts. It enhances wound contraction rate, extracellular matrix production *in vivo* (Mustoe *et al*, 1991; Lanning *et al*, 2000) and the formation of capillary *in vitro* (Sakuda *et al*, 1992). It influences tissue repair by activation of Smad signaling (Dijke *et al*, 2002). It signals through heteromeric complexes of type II and type I transmembrane Ser/Thr kinase receptors, then initiates phosphorylation cascades involving

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; ES, electrical stimulation; TGF-β1, transforming growth factor-β1

receptor-regulated Smads, a co-Smad, and inhibitory Smads.

For decades, investigators have attempted to treat problems related to wound impairment by electrical stimulation (ES). Most of the ES applications are safe and effective. For example, it has long been used in clinics for wound-related pain control (Evans *et al*, 2001; Bjordal *et al*, 2003) and neuromuscular rehabilitation with an excellent safety record (Crevenna *et al*, 2003). Furthermore, electric pulses can accelerate the recovery of diabetic ulcer (Baker *et al*, 1997). In other human studies, ES has been shown to accelerate the recovery of chronic wounds (Gardner *et al*, 1999).

Electroporation is a type of electrical treatment that can enhance cell permeability to allow penetration of macromolecules such as DNA (Banga and Prausnitz, 1998). It has been reported to increase gene transfer in liver and muscle (Aihara and Miyazaki, 1998; Suzuki *et al*, 1998). Delivery of chemotherapeutic agents to cancers using electroporation has progressed to Phase II clinical trial (Heller *et al*, 1997; Heller *et al*, 1999). Due to the complexity of the skin, harsh condition of electroporation, which ranged from electric field strength of 400–2000 V per cm, were mostly used to accomplish effective gene transfer (Titomirov *et al*, 1991; Heller *et al*, 2001). Typical transdermal voltage for drug delivery is 50–150 V (Vanbever *et al*, 1996). High current and voltage applied to the skin can cause tissue damage and electrolysis (Pliquett, 1999). We hypothesized that proper conditions could be found with which both electrical and gene therapies could be additively applied to treat diabetic wound healing without damaging the tissue. The first step of our study was the optimization of parameters such as applied voltage, pulse duration, number of pulses, and types of electrode. We then examined if the optimal condition for TGF-β1 gene transfer at the wound site could also benefit healing due to its electric effect.

Results

Optimization of electroporative condition *In vivo* electroporative gene delivery is commonly accomplished by two kinds of electrodes: calipers and syringe electrodes, a special type of needle electrodes. The design of the syringe electrode has been previously described (Liu and Huang, 2002). To compare caliper and syringe electrodes in the effectiveness on gene delivery, we intradermally injected 40 μg of luciferase reporter gene to C57BL/6 or C57BKS.cgmLepr^{db} mice followed by electroporation using caliper or syringe electrodes. We found that the syringe electrodes were more effective in gene transfer compared with the caliper electrode. In Fig 1*A*, the luciferase gene expression in the syringe-electroporated skin was 10-fold higher compared with the caliper electroporated skin when the same applied voltage and duration (100 V in 20 ms) were used. Therefore, in our further experiment, we decided to use the syringe electrodes.

To optimize the electroporative condition for the syringe electrodes, we tested different applied voltage and found that 100 V for a duration of 20 ms with six pulses was the optimal condition for gene transfer in the skin tissue (Fig 1*B*). Higher voltage did not induce a corresponding increase in the gene expression. Furthermore, when we compared the luciferase gene expression of two skin types (diabetic and normal), the diabetic skin performed 10-fold higher expression compared with the normal skin at these conditions. Bubbles, a sign of electrolysis, were observed surrounding the electrodes in the diabetic skin when 200 V per cm field strength was applied. This observation did not appear in the normal skin, suggesting that the diabetic skin is more sensitive to electroporation in mouse model.

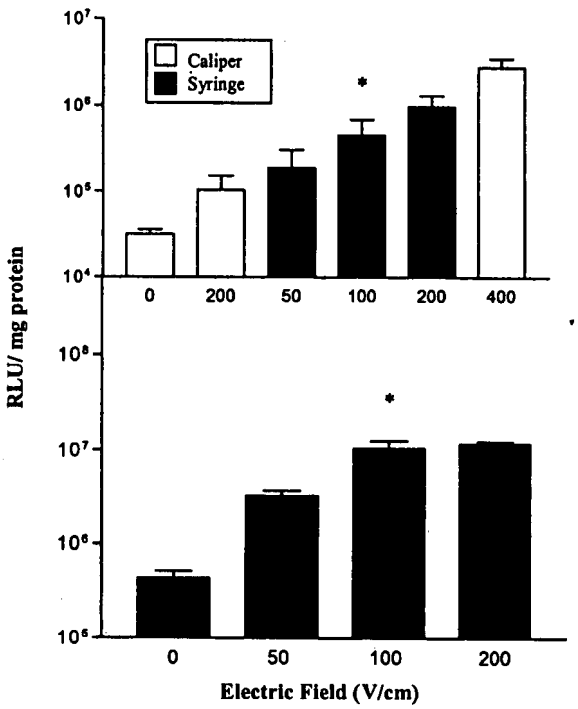

Figure 1
Luciferase activity after electroporation using different electrodes and electric parameters on normal (*A*) and diabetic (*B*) skin. *$p < 0.001$, comparing data of 100 V using syringe electrode with all other conditions; $n = 6$.

Local expression of TGF-β1 at the wound bed Next, we transfected the diabetic skin tissue with our therapeutic gene, TGF-β1, using the optimized condition. To measure the skin transfection by the plasmid, we used an ELISA kit, which was coated with the TGF-β1 receptor to detect human TGF-β1. As shown in Fig 2, intradermal injection of plasmid TGF-β1 gave a significantly ($p < 0.05$) higher expression of TGF-β1 compared with the untreated, electroporation alone or intradermal injection of the empty plasmid. Intradermal injection of plasmid TGF-β1 followed by electroporation produced 2-fold higher cytokine level compared with the intradermal injection of plasmid TGF-β1 alone, suggesting that electroporation enhanced the gene transfer as previously reported (Heller *et al*, 2000). On the other hand, the untreated tissue produced low level of TGF-β1 cytokine. Furthermore, wound treated with intradermal injection of empty plasmid or electroporation alone also produced TGF-β1 cytokine as low as the untreated. These low levels of activity might arise from the cross-reactivity of the antibody with endogenous murine TGF-β1.

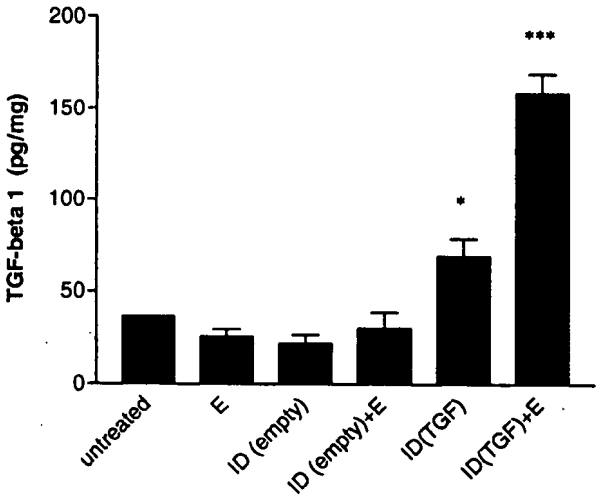

Figure 2
Transforming growth factor-β1 (TGF-β1) cytokine level 24 h after the following treatments: electroporation only (E), intradermal injection of empty plasmid [ID(TGF)] or plasmid with TGF-β1 gene [ID(empty)], electroporation following intradermal injection of plasmid with [ID(TGF)+E] or without TGF-β1 gene [ID(empty)+E]. ***$p<0.001$, comparing treatment of TGF-β1 gene by electric pulses with all other treatments. *$p<0.05$, comparing treatment of TGF-β1 gene by intradermal injection with all other treatments; $n=6$.

Wound-healing parameters Wound healing is a multiple-step process. In order to investigate the progress of wound healing, wound-healing phases such as reepithelialization, wound closure, collagen synthesis, and angiogenesis were examined in the diabetic skin.

Reepithelialization Reepithelialization is a process involving keratinocyte migration followed by keratinocyte proliferation. At day 5 postwounding, hematoxylin & eosin (H&E) staining showed that the leading end of the epithelium (i.e. the epithelial tongue schematically shown in Fig 4*A*) of all wounds migrated towards the center (from left to right in Fig 3). The epithelial tongue (indicated by arrow in Fig 3) moved the fastest in the wound treated with plasmid TGF-β1 injection followed by electroporation. The electric treatment appears to induce the fibroblast migration. As can be seen in Fig 3, cell density in the granulation tissue (schematically shown in Fig 4*A*) in the wound treated with electroporation alone (Fig 3*B*) was higher than in the untreated wound (Fig 3*A*). The majority of the observed cells in the granulation tissue were spindle-like, similar to the shape of fibroblasts. Anti-5-bromo-2-deoxyuridine (BrdU) immunohistochemical staining further showed that the epithelial cells were the most actively proliferated in the electro-TGF-β1 gene-treated wounds. Cell proliferation was also significantly induced by simple plasmid TGF-β1 injection. On the contrary, other treatments produced comparable level of cell proliferation as the untreated.

When we investigated the epidermal cell proliferation at the edge of the wound, the proliferation rate (Fig 4*B*) at region 2 (Fig 4*A*) was higher than at region 1, which is the leading edge of the epithelial tongue. The difference tends to be significant ($p<0.05$) in the actively proliferated wound bed that received the plasmid TGF-β1. In both locations, the wound treated with intradermal injection of the TGF-β1 gene followed by electroporation induced higher proliferation than other treatments. Intradermal injection of plasmid TGF-β1 also induced proliferation significantly ($p<0.05$) in both positions compared with the untreated, electroporation, intradermal injection of the empty plasmid with or without electroporation.

Wound closure Wound contraction is the process to minimize the open area by pulling the neighboring tissue towards the wound center. Myofibroblasts differentiated from fibroblasts generate the contractile force (Jester *et al*, 1999; Feugate *et al*, 2002). It occurs faster than reepithelialization

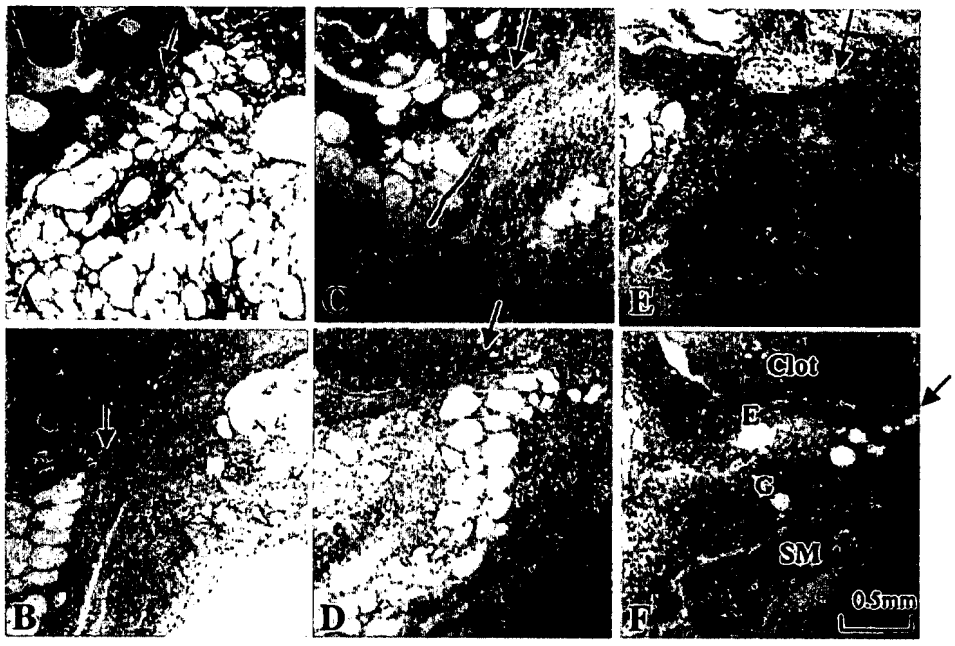

Figure 3
Hematoxylin & eosin staining for wound morphology at day 5 after the following treatments. Wound treated without (*A*, *C*, and *E*) or with electroporation (*B*, *D*, and *F*). No injection of plasmid (*A*, *B*), injection of empty plasmid (*C*, *D*) or plasmid with TGF-β1 gene (*E*, *F*). Epithelial tongue is indicated by black arrow. Epidermal tissue, granulation tissue, and smooth muscle is indicated, respectively, as E, G, and SM. Magnification × 100.

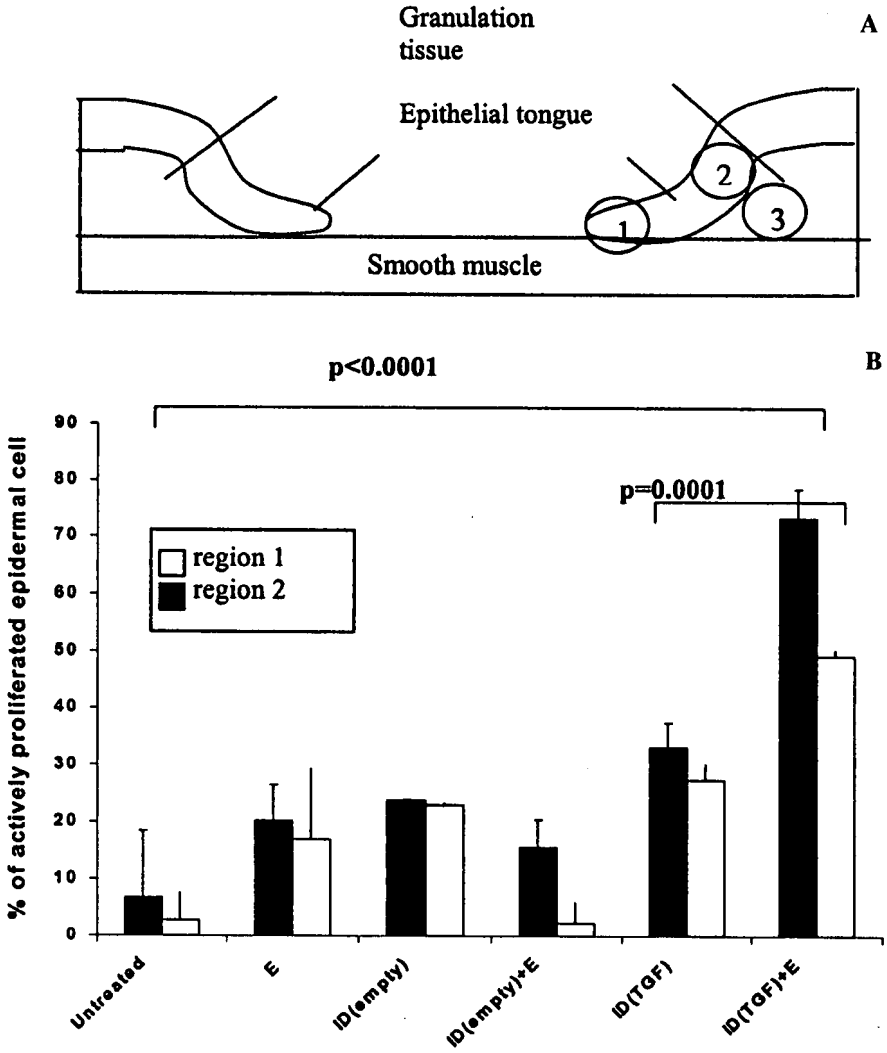

Figure 4
Immunostaining for 5-bromo-2-deoxyuridine (BrdU)-positive keratinocytes at the wound edge of the epithelium at day 3 postwounding following different treatments. (*A*) Schematic drawing of a wound indicating the location of cell counts taken (regions 1 and 2). (*B*) BrdU-positive cell count at region 1 (□) or at region 2 (■) following different treatments; n = 3.

because no cell proliferation is involved. We evaluated the wound-contraction rate by measuring the percentage of wound closure until wound is completely closed at day 14 (Fig 5). When compared with the untreated group throughout the 14 d, all treatments such as electroporation alone, and intradermal injection of plasmid TGF-β1 with or without electroporation induced greater wound contraction. Wound-closure rate was significantly ($p < 0.05$) accelerated at early stages (days 2–5) in the electro-TGF-β1 gene-treated wound bed compared with TGF-β1 gene-treated wound bed. This suggests that additive effect of electroporation combined with TGF-β1 gene treatment on wound closure occurred only in the early phase of wound healing.

Collagen synthesis At day 5 after treating with intradermal injection of plasmid TGF-β1 with or without electroporation, high intensity of collagen was found in the newly formed granulation tissue at the epithelial tongue, with the former being higher than the latter (Fig 6). The collagen organization in the electric-gene-treated wound appeared the most mature, as it resembled the unwounded skin at day 5 (Fig 6*F*, *G*). In addition, we observed that the collagen organization in the smooth muscle layer (white arrow, Fig 6*D*) is more dispersed from the granulation tissue.

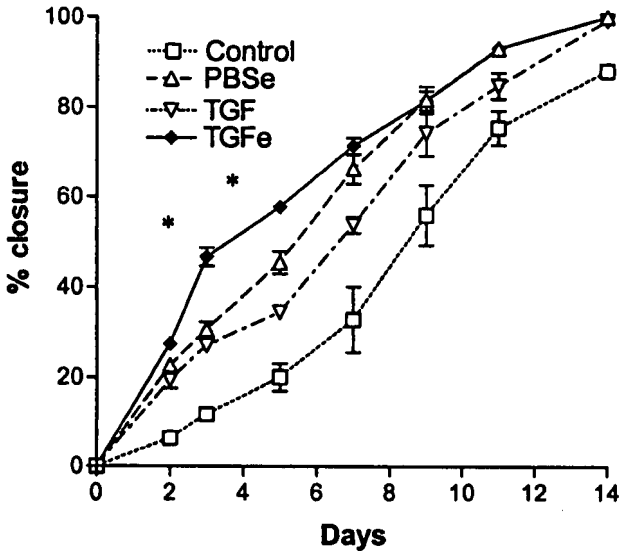

Figure 5
Wound contraction as percentage of wound closure during 14 d after electroporation alone (PBSe), or plasmid transforming growth factor-β1 (TGF-β1) injection with (TGFe) or without electroporation (TGF). Control represents untreated wound. *$p < 0.05$, comparing the treatment of TGF-β1 gene followed by electric pulses with treatment of TGF-β1 gene; n = 12.

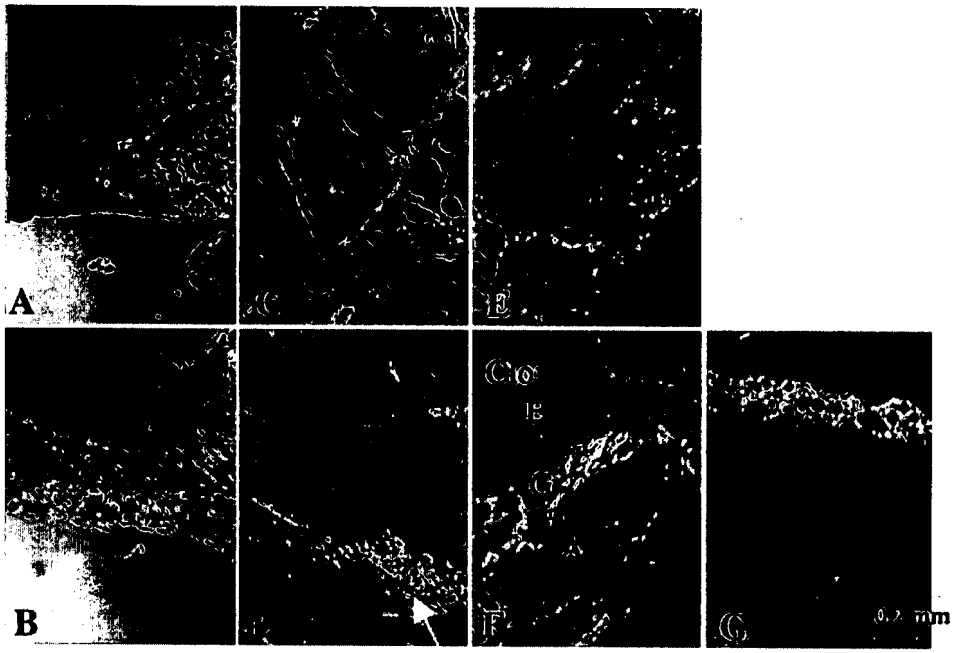

Figure 6
Collagen formation shown by picrosirius staining at day 5 postwounding following the treatment of electroporation (*B*, *D*, and *F*) or without electroporation (*A*, *C*, and *E*). No injection of plasmid (*A*, *B*), injection of empty plasmid (*C*, *D*), or plasmid with transforming growth factor-β1 gene (*E*, *F*). (*G*) Unwounded skin. Collagen is indicated as glowing yellowish orange. Epidermal tissue, granulation tissue, adipose tissue and smooth muscle is marked as E, G, A and SM, respectively. Magnification × 200.

Angiogenesis Angiogenesis is a process for new capillary growth and one of its components is endothelial cell migration (Lingen and Nickoloff, 2001). At day 7, sections of harvested wound bed were stained with anti-factor VIII-related antigen to identify endothelial cells in the newly synthesized granulation tissue in the wound. A higher density of endothelial cells was found in the electro-TGF-β1 gene-treated wound than was found in all other treatments. In the wound bed treated with simple injection of TGF-β1 gene, stained endothelial cells could be observed in one of the three representative fields (black arrow, Fig 7*E*). On the other hand, with electroporation, more intensely stained endothelial cells were observed in all three fields (Fig 7*F*). The number of stained endothelial cells found in different treatment groups is shown in Fig 7*G*. Only electro-TGF-β1 gene therapy appeared to enhance endothelial cell migration significantly ($p<0.05$), suggesting an enhanced angiogenesis.

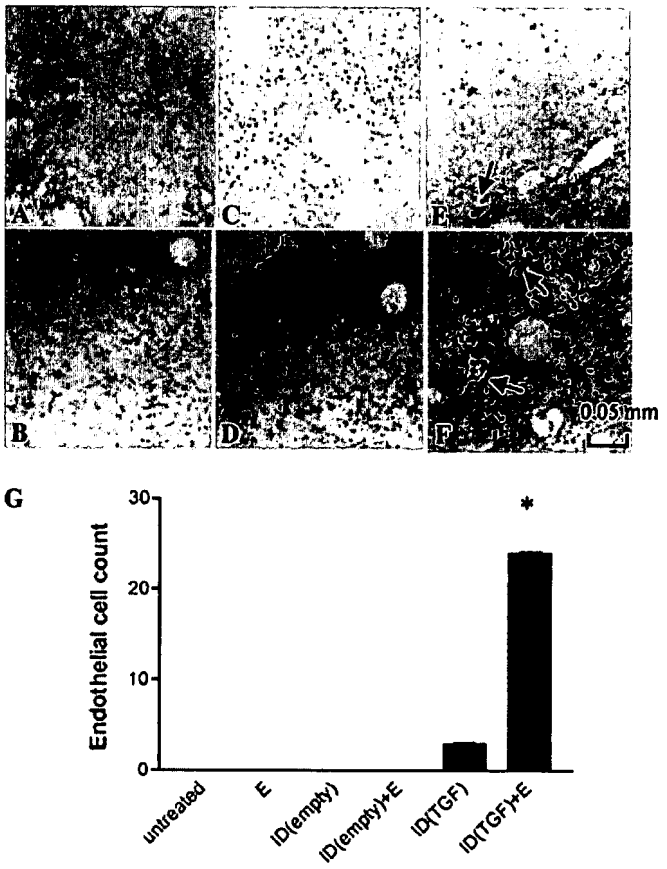

Figure 7
Angiogenesis at the granulation tissue at the center of the wound bed (region 3) at day 7 postwounding following the treatment without (*A*, *C*, and *E*) or with electroporation. (*B*, *D*, and *F*). No injection of plasmid (*A*, *B*), injection of empty plasmid (*C*, *D*) or plasmid with transforming growth factor-β1 (TGF-β1) gene (*E*, *F*). Endothelial cells are indicated by black arrow. Magnification × 400. (*G*) Endothelial cell count in three representative fields. *$p<0.05$, comparing the treatment of TGF-β1 gene by electric pulses with all other treatments.

Discussion

This report demonstrates the potential of the combination of gene and electric therapies to treat diabetic wound healing. For this purpose, we looked for conditions in which both TGF-β1 gene transfer and electrical treatment occurred. We started the experiment searching for the optimal condition for enhancing gene transfer. We applied electric pulses immediately after injection of the luciferase reporter gene. We found that six pulses of 100 V per cm in 20 ms duration with the syringe electrodes was the most effective condition, and accomplished transfection level of approximately 10^7 RLU per mg of protein. To achieve a comparable transfection level, most of the investigators used the electric field 700–1200 V per cm (Heller *et al*, 2001) which are 7-fold higher than our applied electric field. In other words, we used much milder conditions to produce comparable gene transfection. This is important since the diabetic skin is

more sensitive to electoporation than the normal skin in this mouse model. The use of a mild condition was necessary to prevent tissue damage.

Then we studied if our optimal condition could bring about the therapeutic effects of TGF-β1 gene transfer and electrical therapy. We investigated several well-recognized wound-healing parameters such as reepithalization, wound closure, collagen deposition, and angiogenesis with our diabetic excisional wound model. We used the same syringe for the injection of the TGF-β1 gene and the application of six pulses of 100 V per cm in 20 ms duration without withdrawing the needle. In this experimental setting, we found that electric pulses alone could induce cell migration (Fig 3) and wound closure (Fig 5). Simple injection of TGF-β1 plasmid could induce reepithalization, wound closure, collagen deposition, and angiogenesis. As we expected, the application of electric pulses along with the delivery of TGF-β1 plasmid further enhanced all wound-healing parameters. An increase of reepithelialization rate, wound contraction, collagen synthesis, and angiogenesis was found in the wound 5–7 d postapplication of intradermal injection of TGF-β1 followed by electroporation. Not surprisingly, the improvements in reepithelialization rate, collagen synthesis, and angiogenesis were greater than the additive effects of gene and electric treatments. There was a clear synergism between the two treatments.

TGF-β1 is a multifunctional cytokine and the biological response is complicated in wound healing. It is a potent chemokine for fibroblasts. It enhances granulation tissue and collagen formation. On the other hand, TGF-β1 is a potent inhibitor of keratinocyte proliferation *in vitro* (Garlick and Taichman, 1994a; Garlick *et al*, 1996). One possible reason is that the TGF-β1 pathway is a negative feedback mechanism for epidermal growth factor-induced proliferation of human keratinocyte (Yamasaki *et al*, 2003). In a steroid-impaired rabbit wound model, however, application of TGF-β1 enhances reepithelialization (Beck *et al*, 1991). Another study further supported that TGF-β1 acted as an inducer in keratinocyte proliferation *in vivo*. This group showed that overexpression of TGF-β1 promoted epidermal cell growth in a TGF-β1 transgenic model (Fowlis *et al*, 1996). Besides keratinocyte proliferation, TGF-β1 can induce keratinocyte migration by upregulating the synthesis of laminin 5, which has a dual function in keratinocyte adhesion or migration (Decline *et al*, 2003). Taken together, TGF-β1 is beneficial to wound healing. Indeed, the therapeutic effect of plasmid TGF-β1 has been fully documented by our group (Chesnoy *et al*, 2003). Our data is consistent with the previous findings.

ES has been known as a cell migration promoter. Studies showed that electrical field stimulates macrophage, corneal epithelial cells, and fibroblast migration (Brown and Loew, 1994; Cho *et al*, 2000; Wang *et al*, 2003). Our results also showed that electroporation induces cell migration (Fig 3*B*). The induction is associated with an alteration of cell movement including cell crawling and possibly cell rolling without changing the cell morphology (Cho *et al*, 2000). The mechanism of electric field-induced migration is not yet clearly elucidated, but integrin-dependent signaling may be involved in electric field-induced macrophage migration (Cho *et al*, 2000). Furthermore, the electric stimulation also enhances the activation of ERK1/2, a signaling molecule in the MAP kinase pathway (Wang *et al*, 2003).

In our study, we found that the actively migrating keratinocytes at the leading end (region 1, Fig 4*A*) does not proliferate. Instead, most of the proliferation took place in the vicinity of the leading end (region 2, Fig 4*A*). Previous studies reported that reepithelialization is temporally and spatially coordinated: keratinocyte migrate into the wound followed by transiently burst proliferation at the wound margin, and the actively migrating keratinocytes does not proliferate (Garlick and Taichman, 1994b). Thus, our data is consistent with previous findings.

In conclusion, we have proposed an innovative strategy for therapeutic treatment of diabetes-induced wound impairment with a combination of electric and gene therapies that may have a significant implication for clinical applications.

Materials and Methods

Animal Female mice of C57BL/6 or C57BKS.Cg-m+/+Lepr^{db} (type II diabetes) in 7–9-wk old were obtained from Jackson Laboratories (Bar Harbor, Maine). C57BKS.Cg-m+/+Lepr^{db} mice have been used as a model for wound healing in diabetics, especially for studies involving cytokines and growth factors (Greenhalgh *et al*, 1990; Okumura *et al*, 1996). Mice homozygous for the diabetes spontaneous mutation (*Lepr^{db}*) become identifiably obese around 3–4 wk of age. Elevations of plasma insulin begin at 10–14 d and elevation of blood sugar at 4–8 wk. All mice were housed in the animal facility at the University of Pittsburgh. All animal protocols were approved by IACUC of the University of Pittsburgh.

Plasmids Human TGF-β1 cDNA in pcDNA3.1/GS (Invitrogen Corporation, Carlsbad, California) was amplified in TOP10 competent cells (Invitrogen Corporation). The plasmid DNA was isolated by alkaline lysis and purified by ion exchange column chromatography (Qiagen Inc., Valencia, California). Plasmid pNGVL-luc, which encodes luciferase as a reporter protein (National Gene Vector Laboratory, Ann Arbor, MI) was obtained similarly.

Wounding protocol and treatment A total of 36 C57BL/6 and 136 C57BKS.Cg-m+/+Lepr^{db} mice were anesthetized by inhalation of Isoflurane and randomly divided into groups to receive different treatments or control in different experiments. Forty micrograms of plasmid pNGVL-luc (dissolved in 50 μL 1.5 × PBS) intradrmally injected into skin of C57BKS.Cg-m+/+Leprdb or C57BL/6, followed by electorporation using caliper (BTX Gentronics Inc., San Diego, California) or syringe (Liu and Huang, 2002) electrodes for optimization. Intradermal injection of plasmid pNGVL-luc was also done for comparison. For the studies of ELISA and therapeutic effect, two 7 × 7 mm full thickness excisional wounds were created in parallel on the back of each mouse after the mice were anesthetized. Human recombinant plasmid TGF-β1, 30 μg dissolved in 50 μL PBS was intradermally injected to the lateral sides of a wound followed by electroporation using syringe electrode. Control mice received either no treatment, electroporation only, intradermal injection of empty plasmid with or without electroporation or intradermal injection of plasmid TGF-β1 without electroporation.

Measurement of luciferase reporter gene expression Six mice (12 wounds) in each group were euthanized and skin biopsies were collected and homogenized. Luciferase gene expression (activity) was measured using a luminometer. The activity was presented as relative unit per milligram soluble tissue protein (RLU per mg protein).

Local expression of TGF-β1 using immunoassay At 24 h postapplication, three mice (six wounds) in each group were euthanized and wound biopsies were harvested and homogenized with

protease inhibitor (Roche Diagnostics, Indianapolis, IN). TGF-β1 protein concentration was measured with human TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN) following the supplier's protocol.

Wound-closure analysis Six mice in each group were examined. Area of wounds was measured using a caliper at each day, in a total of 14 d, and evaluated as percentage of wound closure using the equation

$$\%\,\text{wound closure} = 100 \times (\text{wound area at day 0} - \text{wound area at day n})/\text{wound area at day 0}$$

Histology Three mice from each group were euthanized and skin biopsies were harvested at days 3, 5, and 7 postwounding. The harvested tissue was formalin fixed and embedded in paraffin. Sections of 4 μm thickness were prepared using a microtome, then deparaffinized, hydrated, and stained with H&E for observing the morphology following Degroat's protocol and picrosirius red staining using a 0.1% picrosirius red solution (Sweat *et al*, 1964).

Cell proliferation using anti-BrdU immunhistochemistry At day 3 postwounding, BrdU (Sigma, St Louis, Missouri) labeling was performed by intraperitoneal injection at a dose of 50 mg per kg at 3 h prior to euthanization. Paraffin sections were taken from specimens at the wound site. The sections were deparaffinized, hydrated, pre-treated with 2 N HCl and trypsin (Sigma). BrdU immunochemical staining was performed by incubation of a rat monoclonal anti-BrdU antibody (Accurate Chemical & Scientific Corp., Westbury, New York) for 18 h at 37°C. Sections were then incubated with biotinylated mouse-adsorbed rabbit anti-rat IgG and were peroxidase labeled with Vetastain Elite ABC Kit (Vector Laboratories, Burlingame, California). The immunoprecipitate was visualized by 3,3′-diaminobenzidine tetrahydrochloride chromogen and Gill 1 × hematoxylin (Fisher Scientific, Pittsburg, PA) counterstain. Sections were observed under a microscope (Nikon, Tokyo, Japan), and positively stained cells were counted in three representative fields at × 200 magnification.

Angiogenesis using anti-factor VIII-related antigen immunohistochemistry Paraffin sections were taken from specimens at the wound site at day 7 postwounding. Factor VIII-related antigen immunochemical staining was performed with incubation of rabbit polyclonal antisera for factor VIII antigen. All other steps were performed the same way as mentioned in the method for anti-BrdU staining. Sections were observed at × 200 magnification.

Statistical analysis Data were expressed as means ± standard deviation (SD) and analyzed by two-tailed Student's *t* test using the PRISM software program (GraphPad Software, San Diego, California). The α value (type I error) adjustment was done by using Bonferroni correction in case of multiple comparisons.

This work was supported in part by NIH grants, DK44935, AR45925, and AI48851.

DOI: 10.1111/j.0022-202X.2004.23309.x

Manuscript received December 9, 2003; revised March 10, 2004; accepted for publication March 24, 2004

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EXHIBIT 3

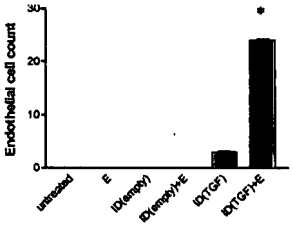
CLINICAL SNIPPETS

Andrea M. Sattinger
Lowell A. Goldsmith

Women Are Catching Up

In the Netherlands, basal cell cancer (BCC) is the most common malignant tumor in pale-skinned individuals. Where a "typical" BCC patient has traditionally been an older male with chronic sun exposure usually on the head and neck, data now show young or middle-aged females who sunbathe in shorter, more intense bursts are increasingly presenting with tumors on the trunk and limbs. When de Vries and coworkers considered hypotheses for this change in the 20th century, they identified increasing leisure time, different cultural mores and patterns of clothes and body exposure, and more affordable travel costs to tropical destinations. J Invest Dermatol 123:634–638, 2004.

Electrical and Gene Therapies for Wound Healing



Exogenous growth factors have been successfully used to accelerate wound healing in humans and animal models. A less expensive alternative, however, is to administer a gene that encodes growth factor. Transforming growth factor (TGF)-β1 is a chemokine for fibroblasts that enhances granulation tissue but is a potent inhibitor of keratinocyte proliferation *in vitro*. Electroporation is also a cell-migration promoter and can enhance cell permeability to allow DNA penetration. The combination of electric pulses and delivery of TGF-β1 plasmid provided an innovative synergism to treat diabetic wound healing in the db/db (diabetic) mouse. The findings by Lee and co-workers may have a significant implication for clinical applications. J Invest Dermatol 123:791–798, 2004.

Tea Tames Weal and Flare

Determining the bioactive mechanisms of essential oils is not an easy task. Tea tree oil (TTO), from the Australian plant *Melaleuca alternifolia*, reduces histamine-induced vascular responses in humans. Khalil and colleagues dissected TTO's effect on inflammatory microvascular changes of rat blisters to identify the active components and the mechanism of action. After histamine injection in human skin, terpinen-4-ol (40% TTO), but not α-terpineol or 1,8-cineole (3% and 2% TTO, respectively), downregulated the developing weal and flare. The rodent and human studies complement the analysis of the mode of action of immunoregulatory preparations on skin inflammation. J Invest Dermatol 123:683–690, 2004.

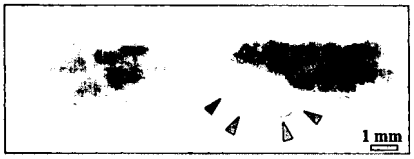
(Photo Tea trees (*Melaleuca alternifolia*) growing at Australian Plantations in Wyrallah, New South Wales, Australia. Courtesy of University of Western Australia.)

Angiogenesis and Wound Healing

While healing, cutaneous wounds have many new capillaries and granulation tissue. Eming and colleagues looked at angiogenesis impairment in patients with normal-healing acute and non-healing chronic wounds. Vascular endothelial growth factor (VEGF)-A is pivotal in the angiogenic response. sVEGFR-1 inhibits VEGF-A and, consequently, angiogenesis. Inducing sVEGFR-1 expression to non-physiologic levels will promote understanding of which factor in wound fluid is responsible for such an induction. Detection of sVEGFR-1 may lead to better assessment of disease severity and progression, and may serve as an indicator of therapeutic efficacy of topical and systemic therapy. J Invest Dermatol 123:799–802, 2004.

The Mystery of Mutating Moles

Many benign and malignant melanocytic lesions exhibit BRAF gene mutations, usually as V599E (changing valine at protein 599 to glutamic acid). Loewe and colleagues examined 324 lesions that were unremarkable at initial visit but showed structure and/or size changes at follow-up. Photo-documentation and



then excision showed 24 of them to be melanomas. The $BRAF^{V599E}$ mutation correlated with sudden onset of lesion growth. Even though histology proved that many samples were benign, nevi with BRAF mutations may still be melanocytic. Since BRAF mutations cause cell proliferation *in vivo*, Sherlock Holmes will need to take the case of proliferating pigmented papules and macules. J Invest Dermatol 123:733–736, 2004.

SCC in RDEB

Recessive dystrophic epidermolysis bullosa (RDEB) is an autosomal recessive disorder characterized by mutations of collagen type VII. Although RDEB leads to numerous serious morbidities, the major cause of death is cutaneous squamous cell carcinoma (SCC) related to the atrophic scars. Arbiser and colleagues, analyzing eight RDEB tumors, identified specific abnormalities in both the p53 and p16ink4a tumor suppressor genes and conclude that these pathways are involved in the pathogenesis of this severe form of SCC. Future animal studies may increase our understanding of the molecular mechanisms driving the carcinogenesis and may lead to urgently needed chemopreventive and treatment strategies, both for RDEB patients and others. J Invest Dermatol 123:788–790, 2004.